

# **Bioconversion of Lignocellulosic Biomass by the Black Soldier Fly in Combination with Solid State Fermentation for Biofuel and Larval Biomass Production**

A Thesis Submitted to the College of Graduate Studies and Research  
in Partial Fulfillment of the Requirements for the Degree of  
Master of Science in the Department of  
Food and Bioproduct Sciences  
University of Saskatchewan  
Saskatoon

By  
Timothy Howdeshell  
2016

## **PERMISSION TO USE STATEMENT**

In presenting this thesis in partial fulfillment of the requirements for a Master of Science degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without written permission. It is also understood that due recognition shall be given to me and to the university of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Food and Bioproduct Sciences  
University of Saskatchewan  
Saskatoon, Saskatchewan S7N 5A8

## ABSTRACT

The digestion of wheat dried distiller's grains with solubles (DDGS) by the black soldier fly larvae (BSFL) was investigated. DDGS is a high carbohydrate, high protein by-product of grain ethanol fermentation. Around ten percent of the dry weight of DDGS is cellulose, and a further five percent is starch which together represents a large fraction of glucans that escaped the ethanol fermentation process. Digestion by BSFL was expected to result in the degradation of DDGS constituent matrices, particularly fiber, which would increase the recovery of glucose from DDGS to be used in further bioethanol fermentation. Additionally, proteins and digestible carbohydrates could be recovered as BSFL biomass. We considered two studies based on this hypothesis: enzyme and microbial investigation in BSFL culture; and microbiological aids in the BSFL degradation of DDGS. Secretion of enzymes from the BSFL into their feed bed can potentially contribute to the insects DDGS digestive capabilities along with symbiotic bacteria associated with the BSFL. The nutrient composition of the BSFL's diet may impact their digestive capabilities. To investigate this hypothesis, and specifically the effect of the fiber content of the feed, we first analyzed enzyme activities in feed beds which varied in cellulose content using food commodities (corn meal, DDGS, and whole milk powder) containing complex matrices to evaluate the effect of cellulose content directly. Enzyme activities in spent larval feeds were shown to decrease as the cellulose content of the feed increased. From these BSFL spent feeds, bacteria associated with BSFL were isolated and included *Lactobacillus plantarum*, *Sphingomonas melonis*, *Pseudomonas sp.*, and *Klebsiella pneumoniae*. These bacteria should have originated from the digestive system of the BSFL, which were hypothesized to play a role in the BSFL's digestive capabilities. The contribution of these isolates in enzyme activities were also analyzed, and *Lactobacillus plantarum* showed the greatest overall enzyme activity based on protease, cellulase, amylase, and lipase assays of the isolated bacteria. Because the digestive capabilities of BSFL were shown to decrease as the cellulose content of their feed increases in this study, we further hypothesized that fermentation of cellulosic materials prior to BSFL digestion can assist the contribution of the larvae in glucose recovery from the cellulosic materials. It is investigated whether the use of solid state fermentation (SSF) could degrade DDGS fiber matrices and improve the overall nutrient profile for BSFL culture and recovery of fermentable sugars. The following fungal strains, chosen for their cellulolytic ability:

*Trichoderma koningii*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Phanerochaete chrysosporium*; and *Lactobacillus plantarum* isolated from the BSFL, were used in SSF of DDGS, prior to larval digestion. SSF was shown to open the DDGS structure; reduce the cellulose content by around 5 percent; decrease the total carbohydrate content by up to 13 percent; and increase the protein concentration by up to 8 percent. While all fungal SSF treatments showed positive changes to DDGS nutrient composition, SSF using *Aspergillus niger* had the greatest effect on DDGS, reducing the carbohydrate content by 13 percent, and increasing the protein content by 8 percent. Following this analysis, we investigated whether fermented DDGS was suitable for larval feeding, and the effect that BSFL digestion had on the fermentable sugar recovery from DDGS. In order to analyze the susceptibility of DDGS cellulose into glucose, a two-step hydrolysis assay was employed. Dilute acid hydrolysis was utilized as an initial hydrolysis, which hydrolyzed primarily amorphous cellulose along with starches and bound glucose into monomeric glucose. Then, the solid matter from dilute acid hydrolysis was subjected to enzymatic hydrolysis as the second step of sugar liberation, where cellulases hydrolyzed the remaining crystalline cellulose into monomeric glucose. Digestion of fermented DDGS by BSFL reduced the dry matter of the substrate and incorporated it into a high protein (37% dwb), high fat (23% dwb) larval biomass. Considering DDGS contains very low fat (2%), the significant fat recovery/yields can be achieved though SSF-BSFL treatment. When untreated DDGS was given to BSFL, the larvae incorporated 43.7 % of dry mass of DDGS; whereas SSF DDGS was incorporated up to 80.4% after the digestion by a combination of SSF and BSFL culture. The relative carbohydrate contents of the substrates were increased during larval digestion which, along with opening of the DDGS constituent matrices during treatments, allowed for increased glucose liberation from the substrates in spent feeds. Optimization of hydrolysis conditions, including higher temperatures, pressure, and substrate loading concentration, further provided large increases in total glucose yields compared to unfermented DDGS (8 % of dry weight of DDGS carbohydrates), particularly for *Lactobacillus plantarum* SSF (16% of dry weight of DDGS carbohydrates).

# TABLE OF CONTENTS

PERMISSION TO USE STATEMENT .....	i
ABSTRACT.....	ii
ACKNOWLEDGEMENTS .....	vi
LIST OF TABLES .....	vii
LIST OF FIGURES .....	viii
LIST OF ABBREVIATIONS .....	xi
<b>1 INTRODUCTION.....</b>	<b>1</b>
<b>2 LITERATURE SURVEY.....</b>	<b>3</b>
2.1 The Need for Alternative to Petroleum Based Fuels.....	3
2.2 Background on Lignocellulosic Ethanol Production.....	5
2.3 DDGS Chemical Profile and Liberation of DDGS Fermentable Sugars .....	14
2.4 The Advantages of Black Soldier Fly Bioprocessing of Plant Biomass .....	15
2.5 BSFL as a Novel Protein Source and Addressing Food Security.....	15
2.6 Rearing and Life Cycle Optimization of the Black Soldier Fly.....	17
2.7 Diet of the Black Soldier Fly.....	20
2.8 Digestive Enzymes Native to the BSF.....	22
2.9 Bacterial Diversity in the BSF.....	24
2.10 Manure and Waste Management with the BSFL.....	27
2.11 Anti-Microbial and Anti-Parasite Actions of BSFL.....	28
2.12 Biodiesel Production from BSFL.....	29
2.13 Solid State Fermentation of DDGS.....	31
<b>3 HYPOTHESES AND OBJECTIVES .....</b>	<b>33</b>
<b>4 STUDY 1: CHARACTERIZATION OF ENZYMES FROM SPENT LARVAL FEED .</b>	<b>34</b>
4.1 Abstract.....	34
4.2 Experimental Approach .....	34
4.2.1 Materials.....	34
4.2.2 Screening of Spent Larval Feeds for General Enzyme Activities.....	35
4.2.3 Isolation of Potential Cellulolytic Symbionts from Spent Larval Feeds .....	37
4.2.4 Lignocellulose Feed Content Effect on Enzyme Activity.....	37
4.3 Results and Discussion .....	38
4.3.1 Screening of Spent Larval Feeds for General Enzyme Activities.....	38
4.3.2 Isolation of Potential Cellulolytic Symbionts from Spent Larval Feeds .....	41
4.3.3 Lignocellulose Feed Content Effect on Enzyme Activity.....	46
4.4 Connection to Next Study.....	52
<b>5 STUDY 2: LIGNOCELLULOSE FEED CONTENT EFFECT ON SUGAR PRODUCTION .....</b>	<b>54</b>
5.1 Abstract.....	54
5.2 Experimental approach.....	55
5.2.1 Materials.....	55
5.2.2 Acid Pretreatment and Enzymatic Hydrolysis of Digested Lignocellulosic Feed.....	55

5.2.3 Solid State Fermentation and Larval Digestion of Lignocellulosic Biomass.....	57
5.2.4 Optimization of Sugar Liberation from SSF and Larval Digested DDGS.....	60
5.2.5 Proximate Analysis of DDGS and Larvae from SSF and Larval Digestion.....	60
<b>5.3 Results and Discussions .....</b>	<b>61</b>
5.3.1 Purpose of Acid Pretreatment and Enzymatic Hydrolysis of Digested Lignocellulosic Feed .....	61
5.3.2 Evaluation of Larval Digestion of Lignocellulosic Biomass without Microbial Pretreatment.....	63
5.3.3 Solid State Fermentation and Larval Digestion of Lignocellulosic Biomass.....	72
5.3.3.1 Evaluation of Fungal Organism and Particle Size Effect on Lignocellulosic Biomass .....	72
5.3.3.2 Larval Digestion of SSF-Treated Lignocellulosic Biomass.....	88
5.3.4 Optimization of Sugar Liberation from SSF and Larval Digested DDGS.....	105
5.3.5 Proximate Analysis of DDGS and Larvae from SSF and Larval Digestion.....	112
<b>6 SUMMARY AND CONCLUSIONS .....</b>	<b>115</b>
<b>7 PROSPECTIVE RESEARCH.....</b>	<b>117</b>
<b>8 REFERENCES CITED.....</b>	<b>118</b>

## ACKNOWLEDGEMENTS

I would first like to extend my sincere gratitude and appreciation to Dr. Takuji Tanaka without whose support, encouragement and patience this accomplishment would not have been possible. I would like to thank him for all of the things he has taught me scientifically, as well as his mentorship in persistence, hard-work, and critical thinking.

I also thank my advisory committees with my deep gratitude and respect to Dr. Robert Tyler, Dr. Vladimir Vujanovic, and Dr. Supratim Ghosh for their academic advice on my thesis and support during my master's.

I thank Dr. Venkatesh Meda for graciously acting as my external examiner.

Special thanks to my lab mates, Oarabile Michael Kgosisejo, Tae Sun Kang, and Yanyu Huang for advice, friendship, positive attitudes, and always great conversations.

I am grateful to Ann Harley, Patricia Olesiuk, and Donna Selby for their help on personal and administrative works.

Lastly, I am also grateful for the love, encouragement and support of my friends and family throughout my graduate studies.

## LIST OF TABLES

<b>Table 2-1</b>	World Production of Ethyl Alcohol (Million Liters).....	4
<b>Table 2-2</b>	Comparison of Enzyme Activity in Salivary Gland and Gut Extracts from <i>Hermetia illucens</i> and <i>Musca domestica</i> using Api-ZYM Kit.....	23
<b>Table 4-1</b>	Bacterial Isolates from Spent Larval Feed Identified by 16S rDNA Sequencing .....	43
<b>Table 4-2</b>	Enzyme Activities of Bacterial Isolates of Spent Larval Feed .....	45
<b>Table 4-3</b>	Change in Larval Parameters during 8 Day Larval Digestion Cellulose Synthetic Feed Blends.....	50
<b>Table 4-4</b>	Protein Concentration of Cellulose Synthetic Feed Blends.....	50
<b>Table 4-5</b>	Enzyme Activity of Cellulose Synthetic Feed Blends.....	52
<b>Table 5-1</b>	System Changes in Control SSF + Larvae.....	64
<b>Table 5-2</b>	Sugar Liberation (% reducing sugar mgs/ dry weight of the carbohydrates in DDGS) of Post-SSF DDGS to Reducing Sugars by Acidic and Enzymatic Hydrolysis .....	75
<b>Table 5-3</b>	Minimum Inhibitory Concentration (MIC) of the Larval Extract Fractions from <i>Hermetia illucens</i> Larvae .....	76
<b>Table 5-4</b>	System Changes in <i>Phanerochaete chrysosporium</i> SSF + Larvae .....	89
<b>Table 5-5</b>	System Changes in <i>Trichoderma koningii</i> BRRL 54330 SSF + Larvae .....	90
<b>Table 5-6</b>	System Changes in <i>Aspergillus fumigatus</i> BRRL 163 SSF + Larvae .....	91
<b>Table 5-7</b>	System Changes in <i>Aspergillus niger</i> BRRL 322 SSF + Larvae.....	91
<b>Table 5-8</b>	System Changes in <i>Lactobacillus plantarum</i> SSF + Larvae .....	92
<b>Table 5-9</b>	Bioconversions of SSF DDGS into BSFL .....	105



## LIST OF FIGURES

<b>Fig. 2-1</b>	Graphical Representation of the Goals of Pretreatment of Lignocellulose .....	8
<b>Fig. 2-2</b>	Consolidation of Biologically Mediated Events in Cellulosic Ethanol Production...	13
<b>Fig. 2-3</b>	Life Cycle of the Black Soldier Fly .....	18
<b>Fig. 2-4</b>	Degradation Rate of Selected Components in the Mixed Medium of RSW (70%) and Rice Straw (30%) after Conversion by BSFL with or without Rid-X (0.35%).....	30
<b>Fig. 4-1</b>	Proximate Analysis of Larval Feed Dry and Wet Basis. ....	38
<b>Fig. 4-2</b>	Alpha-Amylase Activity of Concentrated Crude Protein Extracts Preserved with Glycerol.....	40
<b>Fig. 4-3</b>	Cellulase Activity of Concentrated Crude Protein Extracts Preserved with Glycerol	41
<b>Fig. 4-4</b>	Protease Activity of Concentrated Crude Protein Extracts of Spent Larval Feed (Mean and Std. Dev.).....	42
<b>Fig. 4-5</b>	Larval Parameters of 0% Cellulose.....	46
<b>Fig. 4-6</b>	Larval Parameters of 25% Cellulose.....	47
<b>Fig. 4-7</b>	Larval Parameters of 50% Cellulose.....	48
<b>Fig. 4-8</b>	Larval Parameters of 75% Cellulose.....	49
<b>Fig. 4-9</b>	Larval Parameters of 100% Cellulose.....	49
<b>Fig. 5-1</b>	Process Flow Chart for the Bioconversion of DDGS .....	56
<b>Fig. 5-2</b>	Larval Parameters Control SSF+Larvae .....	63
<b>Fig. 5-3</b>	Cellulose Content of Control SSF+Larvae during 12 Day Larval Digestion .....	65
<b>Fig. 5-4</b>	Crystallinity Index of Cellulose from Control SSF+Larvae during 12 Day Larval Digestion .....	66
<b>Fig. 5-5</b>	Free-Glucose in Spent Feed from Control SSF+Larvae during 12 Day Larval Digestion .....	67
<b>Fig. 5-6</b>	Sugar Liberation by Acid Hydrolysis of Spent Feed from Control SSF+Larvae during 12 Day Larval Digestion .....	68
<b>Fig. 5-7</b>	Sugar Liberation by Enzymatic Hydrolysis of Spent Feed from Control SSF+Larvae during 12 Day Larval Digestion.....	69
<b>Fig. 5-8</b>	Combined Sugar Liberation of Spent Feed from Control SSF+Larvae during 12 Day Larval Digestion.....	71

<b>Fig. 5-9</b>	Mycelium Coverage of DDGS (0-10).....	73
<b>Fig. 5-10</b>	Protruding Hyphae Height (0-10) .....	74
<b>Fig. 5-11</b>	Particle Size Analysis of DDGS .....	78
<b>Fig. 5-12</b>	Cellulose Content of Ground DDGS Fractions (dwb).....	79
<b>Fig. 5-13</b>	Crystallinity Index of Ground DDGS Cellulose.....	80
<b>Fig. 5-14</b>	Protease Activity (Mean and Std. Dev.) of Post-SSF Homogeneous, Coarse, and Fine DDGS Fractions.....	81
<b>Fig. 5-15</b>	Cellulase Activity of Post-SSF DDGS.....	82
<b>Fig. 5-16</b>	Pre-Hydrolysis Sugars from Post-SSF of Homogeneous, Course, and Fine DDGS Fractions.....	83
<b>Fig. 5-17</b>	Acid Hydrolysis Sugar Liberation from Post-SSF of Homogeneous, Coarse, and Fine DDGS Fractions.....	84
<b>Fig. 5-18</b>	Enzymatic Hydrolysis Sugar Liberation from Post-SSF of Homogeneous, Coarse, and Fine DDGS Fractions.....	86
<b>Fig. 5-19</b>	Combined Sugar Liberation from Post-SSF of Homogeneous, Coarse, and Fine DDGS Fractions.....	87
<b>Fig. 5-20</b>	Larval Parameters <i>Phanerochaete chrysosporium</i> SSF+Larvae .....	89
<b>Fig. 5-21</b>	Larval Parameters <i>Trichoderma koningii</i> SSF+Larvae .....	90
<b>Fig. 5-22</b>	Larval Parameters <i>Aspergillus fumigatus</i> SSF+Larvae .....	90
<b>Fig. 5-23</b>	Larval Parameters <i>Aspergillus niger</i> BRRL 322 SSF+Larvae .....	91
<b>Fig. 5-24</b>	Larval Parameters <i>Lactobacillus plantarum</i> SSF+Larvae.....	92
<b>Fig. 5-25</b>	Cellulose Content of SSF+Larvae during 12 Day Larval Digestion .....	94
<b>Fig. 5-26</b>	Crystallinity Index of SSF+Larvae during 12 Day Larval Digestion .....	96
<b>Fig. 5-27</b>	Free-Glucose in Spent Feed from SSF+Larvae during 12 Day Larval Digestion .....	99
<b>Fig. 5-28</b>	Sugar Liberation by Acid Hydrolysis of Spent Feed from SSF+Larvae during 12 Day Larval Digestion.....	100
<b>Fig. 5-29</b>	Sugar Liberation by Enzymatic Hydrolysis of Spent Feed from SSF+Larvae during 12 Day Larval Digestion.....	101
<b>Fig. 5-30</b>	Combined Sugar Yield of Spent Feed of SSF+Larvae during 12 Day Larval Digestion .....	104

<b>Fig. 5-31</b>	Optimized Free-Glucose in Spent Feed from SSF+Larvae during 12 Day Larval Digestion .....	107
<b>Fig. 5-32</b>	Optimized Sugar Liberation by Acid Hydrolysis of Spent Feed from SSF+Larvae during 12 Day Larval Digestion.....	108
<b>Fig. 5-33</b>	Optimized Sugar Liberation by Enzymatic Hydrolysis of Spent Feed from SSF+Larvae during 12 Day Larval Digestion.....	110
<b>Fig. 5-34</b>	Optimized Sugar Yield of Spent Feed of SSF+Larvae during 12 Day Larval Digestion .....	111
<b>Fig. 5-35</b>	Proximate Analysis of DDGS and Larvae from SSF and Larval Digestion.....	114

## LIST OF ABBREVIATIONS

16S rDNA	16S subunit of rDNA
A280, A540, A560	absorbance at the listed wavelength number in nanometers
AACC	American Association of Cereal Chemists
BSF	black soldier fly
BSFL	black soldier fly larvae
CBM	cellulose binding module
CI	crystallinity index
CPE	crude protein extract
DDGS	dried distiller's grains with solubles
DNS	3,5-dinitrosalysilic acid
DWB	Dry-weight basis ( $\text{g Analyte} / (\text{g Sample} * (1 - (1 * \text{Sample Moisture Content}))))$ )
GHF	glycosyl hydrolase family
GRAS	generally recognized as safe
MRS	de Man, Rogosa, and Sharpe microbiological media
rDNA	ribosomal deoxyribonucleic acid
rpm	rotations per minute
RS	resistant starch
RSW	restaurant solid waste
SSF	solid state fermentation
U.S. FDA	United States Food and Drug Administration
VOA	volatile organic acids
WMP	whole milk powder
WWB	Wet-weight basis ( $\text{g Analyte} / \text{g Sample}$ )

# 1 INTRODUCTION

Petroleum based fuels are presently considered a non-renewable fuel source, and will require replacement by sustainable fuels such as biofuels. Lignocellulosic ethanol, a second generation biofuel, does not compete with food chain, increases the agricultural sector and economy, and reduces consumption and reliance on fossil fuels. Commercialization remains elusive due to the recalcitrant nature of the lignocellulose which increases production costs and difficulties. A biotechnology revolution is therefore needed in lignocellulosic ethanol production.

Enzyme treatment of lignocellulose has a variety of potential benefits such as lignin specific hydrolysis, dissociation of lignocellulosic components, and reduction in the crystalline structure of cellulose, all of which increase the fiber's susceptibility towards processing into fermentable sugars and ultimately ethanol. Solid state fermentation (SSF) provides natural enzymes which hydrolyze the constituent polymers and matrices in lignocellulosic materials. This improves the nutrient profile of lignocellulosic materials, and facilitates their greater application for non-ruminant digestion through lignocellulose dissociation and hydrolysis.

Dried distiller's grains with solubles (DDGS) are a nutritious, cost-effective source of proteins and carbohydrates in the form of resistant starch and fiber. Since DDGS is a by-product of ethanol production, utilizing it for cellulosic ethanol production helps to increase the total ethanol realized from the starting grains. The high fiber content of DDGS, up to 44%, makes this feed less than desirable for non-ruminant feeds. SSF may increase the protein content, and reduce the fiber content making it a useful treatment step in the bioconversion of DDGS to higher value ones.

In addition to alternative fuels, the search for novel protein sources is underway due to the rising cost in fishmeal, and a growing, developing world population. The black soldier fly (BSF) *Hermetia illucens* is a non-pest, non-disease vector insect which has high fat and high protein content. The amino acid profile of the BSF is comparable to fishmeal, and has a higher amount of the essential amino acid lysine than DDGS. Rearing methods are well established for culturing the BSF, and research towards life cycle optimization has been fruitful. This has led to the

generation of a cottage industry that bioprocesses organic wastes into high value fly larvae meal which is sold as a high protein livestock feed additive. The fat in larval biomass can be extracted and turned into a high quality biodiesel which further commercializes the insect.

The BSF has powerful digestive enzymes and mouthparts facilitating the digestion and degradation of biomass. Bacteria associated with the BSF have significant positive influence in the behavior and life cycle development, and enhance the abilities of the fly to colonize feed, digest feed, maintain host health, and assimilate nutrients into their bodies through the production of vitamins, nutrients, antibiotics, and digestive enzymes. These associated bacteria can be used as a SSF organism to pretreat feeds for the BSF, helping to facilitate bioconversion into larval biomass.

Because BSF larvae (BSFL) protein has a superior amino acid profile compared to DDGS, and digestion of lignocellulose by the BSFL has the potential to disrupt lignocellulosic structures, it is sensible to bioconvert DDGS into commercially valuable BSF protein. SSF may improve the nutrient profile, digestibility, and recovery of organic matter in DDGS, and therefore could serve as a pretreatment step before larval digestion of DDGS. Moreover, the combined actions of SSF and BSFL digestion on lignocellulose in DDGS may help to increase the conversion of undigested DDGS fibers into fermentable sugars as well.

This research aimed to increase the nutrient profile and digestibility of DDGS through the use of bacterial and fungal SSF for BSFL culture, and to characterize the bioconversion of the substrate into high value BSF larval biomass, followed by sugar production from unutilized DDGS fibers.

## 2 LITERATURE SURVEY

### 2.1 The Need for Alternative to Petroleum Based Fuels

Petroleum based fuels remain the primary liquid fuel used around the world, despite advances in alternatives. The use of petroleum based fuels has contributed significantly to the phenomenon of global warming, and global conflict as countries fight for access to this resource. The search for economical and sustainable alternative fuels has long been a focus of much scientific research. Fuels obtained from the processing of biological material, so called biofuels, reduce the reliance on fossil fuels improving energy security, provide environmental benefits, and enlarge rural communities<sup>1</sup>. Diversification of a country's fuel portfolio can help bring jobs and money into the country as well<sup>2</sup>. The current production of biofuels is relatively low compared to petroleum based fuels primarily due to the high production costs associated with biofuels<sup>3</sup>. The production of biofuels is inherently limited by the amount of biomass which can be generated and/or harvested sustainably<sup>4</sup>. First generation biofuels, produced from food or feed grains, compete with food production leading to higher food prices. There are also ethical concerns with diverting food suitable for human consumption to produce fuels for primarily wealthy countries. Second generation biofuels, produced from waste or non-food biological material such as plant biomass, do not compete with food production however<sup>5-8</sup>.

Plant biomass in particular has been identified as the only feasibly sustainable source of fuels and materials<sup>9</sup>. Biodiesel and bioethanol are two major types of biofuels which can be produced from plant biomass. Ethanol production in general has increased substantially over the past decade, and as of 2012 was reported to be around 107,000 million liters a year (**Table 2-1**). Much of the bioethanol is currently produced from glucose containing crops such as sugar cane, used extensively in Brazil, and high starch content crops, such as corn. These crops are preferentially utilized due to the readily-hydrolyzed glucose polymers, and the abundance of glucose fermenting organisms commercially available. The most abundant natural form of polymerized glucose in nature is stored as cellulose, which is often intertwined with lignin and

**Table 2-1 World Production of Ethyl Alcohol (Million Liters), (Modified from Renewable Fuels Association, 2013)<sup>10</sup>.**

Region	2012	2011
<b>North &amp; Central America</b>	52117.55	54515.00
<b>South America</b>	21955.39	21849.02
<b>Brazil</b>	21111.24	21097.01
<b>Europe</b>	4311.58	4420.00
<b>Asia</b>	3603.71	3367.88
<b>China</b>	2100.90	2100.00
<b>Canada</b>	1699.65	1750.00
<b>Australia</b>	268.76	330.09
<b>Africa</b>	158.99	145.02
<b>Total</b>	107327.80	109574.00

hemicellulose collectively referred to as lignocellulose. In 1994 Ballerini *et al.* concluded that, although the cost to produce lignocellulosic ethanol remained much higher than petroleum based fuels, if pretreatment steps were sufficiently optimized and the cost-exempt status of biomass derived ethanol was maintained, it could compete with gasoline at assumed crude oil prices of US \$50 a barrel<sup>11</sup>. Optimization of pretreatment steps would include complete substitution of lactose by pentose hydrolysate as the primary carbon source for enzyme production, recycling enzymes at least partially, inclusion of pentose co-fermentation, and increased by-product use<sup>11</sup>. Fast-forwarding to 2010, Service *et al.* reported that with current technologies, the cost to produce lignocellulosic ethanol (\$0.75/L) was almost double that of the market price of oil (\$0.48/L), and much of the optimism about cellulosic ethanol had diminished<sup>12</sup>. There yet remain opportunities for further optimization of the proposed process, which is made difficult due to the recalcitrance of the lignocellulosic biomass. The discovery/design and utilization of novel enzymes systems, the development of inhibitor tolerant enzymes and microorganisms, and optimized process integration have all been identified as promising cost reducing endeavors<sup>13</sup>. This precludes the need for a biotechnology revolution surrounding bioethanol, particularly for enzymes and microorganisms<sup>14</sup>.



## 2.2 Background on Lignocellulosic Ethanol Production

Lignocellulosic biomass is organic material derived from living organisms which contains cellulose, hemicellulose, and lignin. The relative concentration of these components varies between different biomass substrates. Cellulose is a homopolymer; a polymer comprised of only 1 monomer, of D-glucose in  $\beta$ -1,4 linkages. The degree of polymerization from higher order plants is around 14,000 monomer units<sup>15</sup>. Large amounts of intra- and inter-molecular hydrogen bonding leads to the glucose polymer existing in highly ordered crystalline states with a less ordered amorphous regions dispersed throughout<sup>16-19</sup>. A high degree of crystallinity, indicating that a large percentage of the cellulose microfibrils are in an ordered crystalline state, gives cellulose fibrils a highly inelastic structure, and a tensile strength equaling that of steel<sup>20</sup>. The highly packed, strong structure imparts resistance to hydrolysis. The two structural features of cellulose which have the largest impact on the rate and extent of enzymatic hydrolysis are the degree of crystallinity and surface area<sup>21-25</sup>. A low degree of crystallinity, wherein a large percentage of cellulose microfibrils are in an unordered or amorphous state with respect to each other, is associated with a weakened cellulose structure.

Cellulose is naturally synthesized as individual molecules, which are small, linear chains of glucosyl residues, which then self-assemble at the site of biosynthesis<sup>26</sup>. There is evidence that association with hemicellulose helps to regulate this self-assembling process<sup>27</sup>. Approximately thirty of these cellulose molecules are combined together as bundles of fibers known as elementary fibers or protofibers, which are aggregated into larger structures called microfibrils. Microfibrils are assembled into the familiar cellulose fiber. Adjacent sheets of cellulose fibers are held together by weak van der Waals forces in cellulose I, the most abundant form of cellulose found naturally. Van der Waals forces, which are a comparatively weak force individually, are numerous in cellulose I, and the overall effect is a significant contribution to the crystalline structure of cellulose. The packing is so tight that even water molecules are excluded from between microfibrils<sup>28</sup>.

In addition to crystalline and amorphous regions, cellulose fibers contain many irregularities including kinks or twists of the microfibrils, and/or voids including micropores, large pits, and capillaries<sup>21,29,30</sup>. This makes the surface area greater than a theoretical polymer which is perfectly smooth at the monomeric level. These structural irregularities inherent in the structure allow for the hydration of cellulose particles in aqueous solution at least partially. The

external surface area of the cellulose particle is determined by its shape and size, while internal surface area is determined by the cellulose fiber's capillary structure<sup>31</sup>. When considering the internal surface area of cellulose's impact on enzyme hydrolysis, the porosity of the cellulose particle is of concern as size exclusion of the enzyme contributes to a reduction in its functional activity<sup>31</sup>. The size of the voids may be large enough to allow for penetration by many large molecules<sup>18,32</sup>. The increase in specific surface area has been shown to increase almost linearly with the extent of enzyme adsorption<sup>22</sup>.

Both the rate and extent of hydrolysis increase with the increase in surface area<sup>33</sup>. The higher the degree of crystallinity, in other words the higher the degree of packing among primary constituents, the less susceptible the particle is to hydrolysis. Higher degrees of crystallinity also are inhibitory to enzyme adsorption, further reducing the hydrolysis rate<sup>34-36</sup>. The method of Segal *et al.*<sup>37</sup> has been used extensively and relies on X-rays to calculate the crystallinity index, which is a measure of the percent of crystalline regions in the total particle. In research testing of celluloses a variety of purified celluloses are used, but differences in their structure can have an impact on the results of the study. The following brief overview is reviewed in Lynd *et al.*<sup>38</sup> Hollocelluloses, produced from the delignification of lignocellulosic biomass, contain large amounts of hemicelluloses. Microcrystalline celluloses are produced using dilute acid hydrolysis. The dilute acid hydrolysis process removes hemicelluloses as well as much of the amorphous cellulose regions in the fibers leaving nearly pure cellulose. Different microcrystalline celluloses primarily differ in their particle size distributions, and this can impact the hydrolysis rate. Lastly carboxymethylcellulose, an ether of cellulose, has found widespread use due to its high solubility which standardizes cellulase activity testing.

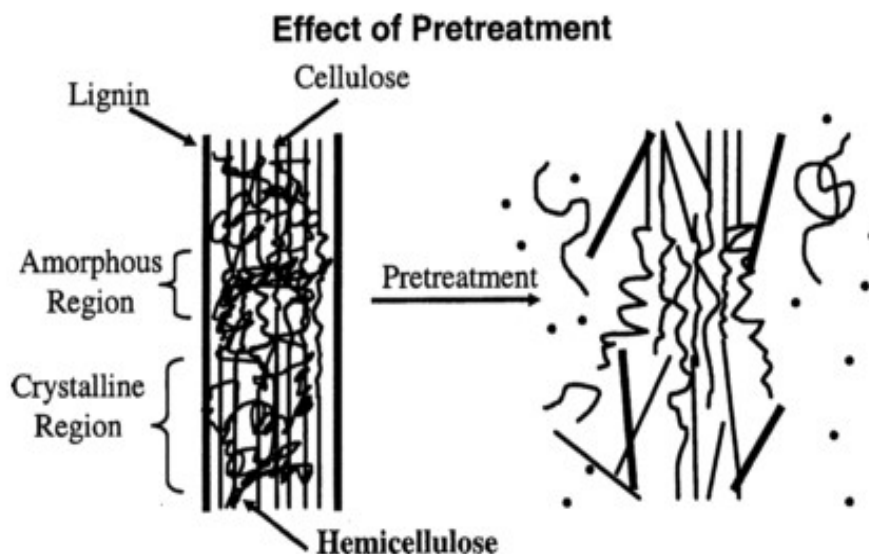
Hemicelluloses are alkali-soluble (primarily heteropolymers) polysaccharides which can be precipitated from aqueous solution by alcohol, and which are more easily hydrolyzed than cellulose by mineral acids<sup>39</sup>. They comprise 15-35% of plant biomass typically and can consist of the following: pentoses such as  $\beta$ -D-xylose, and  $\alpha$ -L-arabinose; hexoses such as  $\beta$ -D-mannose,  $\beta$ -D-glucose, and  $\alpha$ -D-galactose; and/or uronic acids such as  $\alpha$ -D-glucuronic,  $\alpha$ -D-4-O-methylgalacturonic and  $\alpha$ -D-galacturonic acids. Hemicelluloses are broken down into their component monomers upon hydrolysis. Similarly to cellulose hydrolysis, hydrolysis of hemicellulose is accomplished in microorganisms through the production and release of a cocktail of similar enzymes<sup>40</sup>. The primarily heteropolymeric nature of hemicelluloses results in

poor fermentation yields, and is currently the largest polysaccharide fraction wasted in pilot and demonstration ethanol production plants<sup>41</sup>. This presents unique opportunities for biotechnology and the engineering of microorganisms to contain unique genotypes, either through recombinant DNA technology<sup>42-45</sup> and/or genetic engineering<sup>45-49</sup> has been identified as the best solutions to reduce the economic obstacles preventing commercialization of lignocellulosic ethanol<sup>41</sup>.

Lignin is a complex, cross-linked polymer of *p*-hydroxyphenylpropanoid monomers linked primarily by ether or direct carbon-carbon bonds. Both of these bonds are very difficult to hydrolyze. During the lignin polymerization process lignin becomes covalently linked to hemicellulose, in addition to cross-linking between lignin monomers. The degradation of lignin by microorganisms is primarily accomplished by fungi, and most efficiently by white-rot fungi. It occurs through a secondary metabolic process in the presence of low nutrient nitrogen and an additional carbohydrate source such as glucose or cellulose. An extracellular lignin peroxidase in the presence of hydrogen peroxide degrades lignin by oxidative cleavage reactions of the backbone, and monomer units. A free radical mechanism brought about by this lignin peroxidase activity is responsible for lignin degradation. Other enzymes include Mn-dependent peroxidase and laccase<sup>50</sup>.

The fundamental steps in biomass processing for ethanol production are size-reduction and pretreatment (sometimes considered one step), hydrolysis, fermentation, and distillation to obtain the primary end product of ethanol. The crushing step has the function of size reduction of the native biomass, which is particularly relevant for woody biomass feedstocks, and is accomplished by chipping or milling. Size-reduction increases the surface area, and the accessibility for pretreatment processes. The obstacles to this processing step are as follows: the collection which is determined by the type of biomass collection, the efficiency of the collection equipment, and environmental restrictions to prevent soil erosion, soil productivity, and carbon levels; the transportation of the collected biomass which is effected by the distance from the processing plant, the amount of biomass to be transported, and the bulky nature of the biomass which prevents inefficient packing; and lastly, the storage of biomass due to spatial restrictions if the biomass is stored at the processing plant.

A schematic of the structure of lignocellulose, and the goals of pretreatment of lignocellulose are shown in **Fig. 2-1**. The pretreatment step has the general functions of destroying the lignin shell which protects the cellulose and hemicellulose, decreasing the degree



**Fig. 2-1 Graphical Representation of the Goals of Pretreatment of Lignocellulose.**

Reprinted from Zhang B, Shahbazi A (2011) Recent Developments in Pretreatment Technologies for Production of Lignocellulosic Biofuels. *J Phylogenetics Evol Biol* 2:108. doi: 10.4172/2157-7463.1000108, an open-access article distributed under the terms of the Creative Commons Attribution License.

of cellulose crystallinity, and increasing the porosity of the feedstock to facilitate enzymatic hydrolysis. Pretreatment methods, from either the same or different categories, are frequently combined to enhance the effectiveness of the pretreatment step<sup>52,53</sup>. Each individual pretreatment method, of which many have been extensively researched, has inherent advantages and disadvantages. One of the main problems with the pretreatment and hydrolysis of lignocellulosic materials is the variations in the content of lignin and hemicellulose among feedstocks. There are lots of factors influencing this variability including crop type, crop age, harvesting method, and others. This means that no single pretreatment method can be generically applied to all feedstocks<sup>54</sup>. Research trends to improve lignocellulose biomass include the production of genetically engineered plants containing a higher carbohydrate composition, modified plant structures to facilitate mild pretreatment options, and the utilization of hemicellulases (often referred to as pentoses or C-5 sugars)<sup>7</sup>. No one pretreatment method is universally the best. Criteria for effective pretreatment have been established as the following: it avoids size reduction, preserves hemicellulose monomers, limits the formation of inhibitors resulting from degradation of substrates, requires minimal energy inputs, and is cost-effective<sup>55</sup>. Other factors contribute to the selection of a pretreatment method including the ease of high value-added co-

product recovery, the pretreatment catalyst and its ease of recycling, waste treatment concerns, the ease of operation, the cost of downstream processes, and cost tradeoffs between capital, operating, and biomass costs<sup>56,57</sup>. Pretreatment has generally been grouped into three categories: physical, biological, and chemical pretreatment.

Physical pretreatment options include uncatalyzed steam explosion<sup>52,53,58</sup>, and liquid hot water/pyrolysis<sup>59-62</sup> which have the added benefit of hydrolyzing the component as well as reducing the biomass particle size. Uncatalyzed steam explosion is recognized as one of the most cost-effective pretreatment options<sup>63</sup>. In uncatalyzed steam explosion, biomass is saturated with steam or water under highly elevated pressures. The pressure is rapidly reduced resulting in an explosive expansion of the water.

Chemical pretreatment is the most extensively studied among the pretreatment types, and includes numerous methods including the following: catalyzed steam explosion, dilute acid, concentrated acid, oxidative delignification, alkaline, ammonia freeze/ fiber explosion (AFEX), organosolv, pH controlled liquid hot water, CO<sub>2</sub> explosion, ozonolysis, and ionic liquids pretreatment<sup>63,64</sup>. Concentrated acid hydrolysis disrupts the hydrogen bonds which hold the cellulose chain together, and converts the particle to an entirely amorphous state. The cellulose then forms a gel with the acid and becomes extremely susceptible to hydrolysis achieved through dilution with heated water. Concentrated sulfuric acid hydrolysis of cellulose for ethanol production has been successfully commercialized previously, but only in times of war when economic competitiveness could be ignored<sup>65</sup>. Dilute acid hydrolysis uses acid such as H<sub>2</sub>SO<sub>4</sub> and HCl, and requires temperatures of 200-240°C at 1.5% acid concentrations to hydrolyze crystalline cellulose. There is unavoidable degradation of glucose into hydroxymethylfurfural (HMF), and other undesirable products. Xylose is also unavoidably degraded into furfural and other inhibitory byproducts through dilute acid hydrolysis. Variations on the acid hydrolysis application have been proposed. Hamelinck *et al.*<sup>66</sup> utilized a novel two-stage hydrolysis setup in which the first stage was under milder conditions of 190°C, and 0.7% acid for three minutes to recover the pentoses from the more readily hydrolyzed hemicellulose. This was followed by a second stage under more harsh conditions of 215°C, and 0.4% acid for three minutes to recover the hexoses which resulted in a 50% glucose yield. A variant to the above method is the use of very dilute acid concentrations at extremely high temperatures, referred to as the auto-hydrolysis approach, which has been applied to sawdust<sup>67</sup>. Concentrated acid hydrolysis containing 30-70%

acids have higher glucose yields around 90%, but the amount of acid required is not economically favorable<sup>66</sup>.

Biological pretreatment has low energy requirements and mild environmental conditions, but most processes are too slow which limits their industrial application. Many white-rot fungi possess delignifying abilities, and have been utilized for the production of ligninases as well as for lignocellulose degradation<sup>50</sup>. Lee has reported on the main microorganisms which produce ligninases, and the fermentation processes for producing them by both submerged culture and solid-state fermentation<sup>50</sup>.

There are five key areas for future research in lignocellulosic ethanol: (1) reduction of water and chemical use; (2) recovery of carbohydrates and value-added co-products to improve economic feasibility; (3) development of clean delignification which yields benefits for co-fermentation of pentoses and hexoses thereby improving the economic costs of the process; (4) achieving deeper understanding of the fundamental relationship between the biomass substrate and hydrolysis enzymes and the pretreatment mechanisms; (5) reducing the production of inhibitory products such as furfural, 5-HMF, and acetic acid<sup>63</sup>.

While the chemical methods are a combination of exposure and hydrolysis of cellulose, biological and physical methods are followed by hydrolysis process to break down the polysaccharides into their constituent monomers. Hydrolysis can be accomplished by acid and/or enzymatic hydrolysis. Acid hydrolysis is faster, while enzymatic hydrolysis requires lower temperatures, higher monomer yields, is more environmentally friendly, and does not produce glucose degradation products. The general reaction pathway for enzymatic hydrolysis is that crystalline cellulose is converted to cellulose by endocellulases/endo- $\beta$ -glucanases. That cellulose is converted to cellobiose or cellotetrose by exocellulases/exo- $\beta$ -glucanases such as 1,4- $\beta$ -D-glucan cellobiohydrolase and 1,4- $\beta$ -D-glucan glucohydrolase. Finally, cellobiose or cellotetrose is converted to glucose by cellobiases/ $\beta$ -glucosidases<sup>68</sup>. Cellulases need to be bound to the surface of their substrate in order for hydrolysis to occur. Enzymatic cellulose hydrolysis therefore occurs at much slower rates compared to enzymatic hydrolysis of other biopolymers such as starch<sup>69</sup>. Enzymatic hydrolysis of cellulose happens through a complex set of reactions involving several steps<sup>18,19,22,31</sup>: (1) the transfer of cellulases from the bulk aqueous phase to the surface of the cellulose, (2) adsorption of endocellulases/endo- $\beta$ -glucanases and formation of enzyme-substrate complexes, (3) hydrolysis of cellulose, (4) transfer of cellodextrins, glucose

and cellobiose, from the surface of the cellulosic particles to the bulk aqueous phase, (5) hydrolysis of cellodextrins and cellobiose to glucose in the aqueous phase by cellobiases/ $\beta$ -glucosidases. These steps are influenced by the structure of the cellulose, the mode of interaction between the enzymes and substrates, the properties of the enzymes used, and the susceptibility of the enzymes to inhibition<sup>35,70</sup>.

Endoglucanases hydrolyze the internal glucosidic bond in the polymer chain at random locations. They work well against amorphous cellulose, water-soluble cellulose derivative, and produce glucose, cellobiose, cellotriose, and other oligomers. They are not as active against highly crystallized substrates or regions of the polymer<sup>71</sup>. Exoglucanases attack the end of the cellulose polymer at the non-reducing end and produce glucose and cellobiose<sup>71</sup>. Cellobiohydrolases are more active against highly crystalline celluloses and cellulose regions than are endoglucanases, but they are less active against water-soluble cellulose derivatives such as carboxymethyl cellulose<sup>71</sup>. Exoglucohydrolases show high activity against soluble cellodextrins. Due to the variability among enzyme activities towards cellulose hydrolysis products it is necessary for organisms to produce enzyme cocktails to achieve high glucose yields. In fungal cellulose hydrolysis production of all three functional classes of cellulases (endo-, exo- and glucosidases) is required, and synergism is observed when used concurrently<sup>31,72</sup>. Interestingly, exoglucanases may not be required for all organisms to hydrolyze cellulose, and production of that enzymatic class is relatively rare in bacteria<sup>68,73</sup>.

Many organisms produce several isozymes which may differ in the nature and degree of glucose hydrolysis and/or the primary amino acid sequence<sup>68,74</sup>. Additionally, other organisms use different enzymes in the terminal depolymerization of cellulose including cellobiose hydrolase, quinone oxoreductase, cellodextrin phosphorylase, and cellobiose phosphorylase<sup>68</sup>.

*Trichoderma reesei* is a mesophilic fungus which has been extensively studied and utilized for cellulose hydrolysis. *T. reesei* produces a cocktail of cellulases including at least two cellobiohydrolases, five endoglucanases,  $\beta$ -glucosidases, and hemicellulases<sup>69</sup>. Glucose and cellobiose are inhibitors of the enzyme cocktail produced by *T. reesei*<sup>19,35,75-78</sup>. In particular, cellobiohydrolases are inhibited by cellobiose and additional enzyme needs to be added other than what is produced by *T. reesei*. *T. reesei* is the most efficient producer of endo- and exoglucanases<sup>79</sup>, but does not produce a sufficient amount of  $\beta$ -glucosidases<sup>80</sup>. *Aspergillus* strains are known to be good producers of  $\beta$ -glucosidases<sup>81</sup>. *Aspergillus niger* was shown to produce

decent cellulase yields on SSF of wheat bran over a wide range of temperature, pH, and moisture levels<sup>82</sup>.

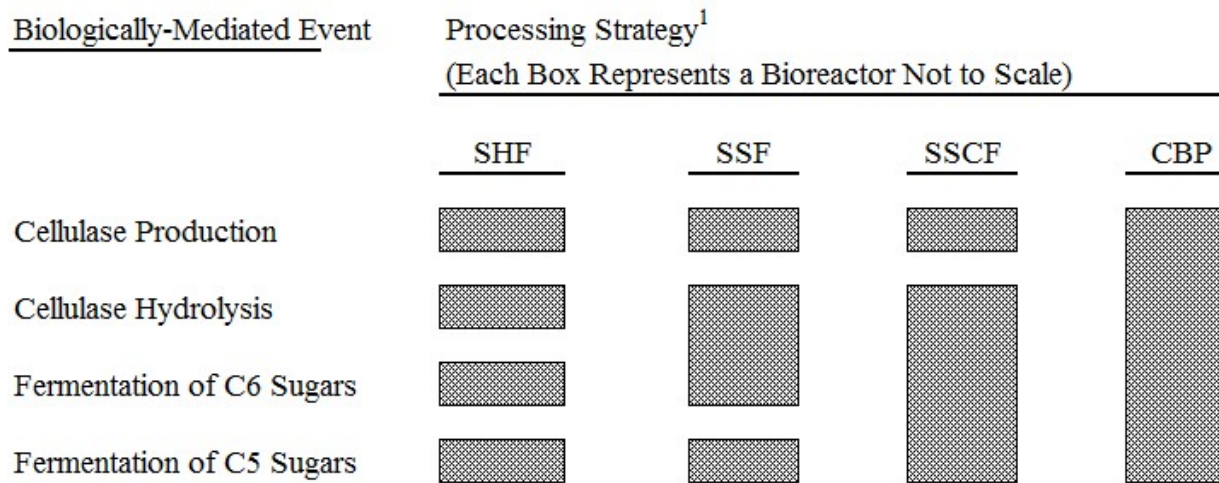
The white-rot fungi *Phanerochaete chrysosporium* is capable of degrading lignin through the production of lignin peroxidases and manganese peroxidases, while leaving the cellulose relatively undisturbed<sup>83</sup>. Since lignin is an indigestible material, and *P. chrysosporium* increases the relative cellulose content of substrates, it represents an attractive organism for biofuel applications. The major challenge in using this organism for enzyme production is the instability of its productivity. This is largely due to a poor understanding of its regulatory mechanisms in response to different nutrient sources in the substrate<sup>83</sup>. *P. chrysosporium* also produces large amounts of protease when low lignin conditions are present<sup>84</sup>. Optimization of the design of cellulose mixtures from various sources in order to maximize the glucose yield has been analyzed<sup>85</sup>.

In addition to fungal fermentation, bacterial SSF has been used as a way to add probiotics to foods, as well as to change to nutritional and functional properties of foods. Fermentation of whole oats with *Lactobacillus plantarum* increased the titratable acidity in the oats through the production of lactic acid, a kind of carboxylic acid (short-chain fatty acid). This helps to prevent contamination of the substrate with undesirable pathogens during the fermentation period. SSF with *L. plantarum* increased the free amino nitrogen by 39% compared to non-fermented oats, and showed no change in soluble dietary fiber or  $\beta$ -glucan content<sup>86</sup>. *L. plantarum* can be used in co-SSF with some fungal strains, and improves the protein content and protein bioavailability of fermented substrates<sup>87</sup>.

The alcohol fermentation step is the final biotechnological step in lignocellulosic biomass processing, and the primary function is to convert pentoses and hexoses into ethanol using microbes. There are many organisms which ferment hexoses to ethanol, but few organisms ferment pentoses and those which are capable, do so at a much slower rate compared to hexose fermentation. Research efforts have been undertaken to incorporate pentose fermenting genes into current hexose fermenting organisms at high conversion levels. This process is referred to as co-fermentation. The recovery and separate fermentation of pentoses must be utilized to obtain the greatest conversion of glucose to ethanol. *Zymomonas mobilis* genes have been introduced into xylose fermenting organisms *Escherichia coli*<sup>88</sup> and *Klebsiella oxytoca*<sup>89</sup>. Incorporation of arabinose fermentation through recombinant DNA technology has also been strong research area



as arabinose is another major component of hemicellulose. The study of enzyme systems is remarkably complex however as cellulases are produced as an enzyme cocktail exhibiting synergistic effects acting upon a multi-component substrate where both the enzyme and substrate composition varies by source and organism<sup>90</sup>. The end goal of these research efforts is to realize a system which incorporates all aspects of production into one reaction mixture which has been



<sup>1</sup>SHF: Separate Hydrolysis and Fermentation

SSF: Simultaneous Saccharification and Fermentation, Separate Pentose Fermentation

SSCF: Simultaneous Saccharification and Co-Fermentation

CBP: Consolidated Bioprocessing

**Fig. 2-2 Consolidation of Biologically Mediated Events in Cellulosic Ethanol Production**

(Adapted from Lynd, 1996)<sup>90</sup>.

dubbed consolidated bioprocessing (CBP)<sup>90</sup>. **Fig. 2-2** shows the various types of solid state fermentation, and how they relate to CBP.

Following fermentation, the ethanol can be recovered from the fermentation broth by distillation, or distillation combined with adsorption, or filtering including drying using salt or lime, addition of an entrainer, molecular sieves, membranes, and pressure reduction<sup>91-93</sup>. The residual solid fraction left over from distillation which includes ash, organism debris, lignin, enzymes, residual cellulose and hemicellulose, and other materials can be recovered as a solid fuel, or further converted to other value-added products.

### 2.3 DDGS Chemical Profile and Liberation of DDGS Fermentable Sugars

A review of the chemical composition of DDGS found that its composition varies based on the processing conditions at the plant of manufacture: crude protein (26 – 37.1%); fat (9.1 – 14.1%); ash (3.7 – 8.1%); acid detergent fiber, a measure of lignin plus cellulose (11.4 – 20.8%); and neutral detergent fiber, a measure of total lignocellulose (33.1 – 43.9%)<sup>94</sup>. During the dry-grind ethanol process a majority of the starch is converted to glucose and then ethanol, and other components experience a three-fold concentration.

Despite that, DDGS still contains a large amount of starch which has escaped either granular starch hydrolysis, or conventional jet-cooking and fermentation processes. Up to 18% of the dry weight of DDGS is residual starch, most of which are in the form of resistant starches (RS). Some starch granules are encapsulated in cells of grain kernel or embedded in protein matrix after milling and thus were physically inaccessible to amylases (RS1). The crystalline structures of native starches were not completely degraded by amylases, retaining some of their structure (RS2). Retrograded starch molecules (RS3), complexes of starch with other non-fermentable components (RS4), and starch-lipid complexes (RS5) were also present in DDGS. Starch which escaped granular starch hydrolysis was mainly RS1 and RS2, whereas that from jet-cooking contained all RS (RS1–RS5)<sup>95</sup>. More RS reduces the ethanol recovery, lowering the efficiency of the process, but treatment of DDGS can liberate glucose. Dilute acid hydrolysis can hydrolyze certain RS types as well as disrupt matrices or complexes which may trap or bind starch, and SSF has the potential for the same through enzymatic action. These sugars can then be incorporated into the upstream fermentation process, increasing the total ethanol yield from the starting grains.

Dilute acid hydrolysis can also liberate lignocellulosic and bound sugars from DDGS, the most industrially important of which is glucose which is derived from cellulose in lignocellulose. Lignocellulose in DDGS is composed mainly of hemicellulose, a complex polymer made of five carbon sugars such as xylose and arabinose. Hemicellulose degradation rates are much higher than cellulose, around 1500 times faster<sup>96</sup>, due to weaker glycosidic bonds in hemicellulose than cellulose. Using dilute acid hydrolysis on corn DDGS produced xylose, arabinose, and glucose in order of decreasing abundance. Reaction temperature, time, and acid concentration had significant effects on xylose and arabinose yields, while glucose was affected only by temperature and acid concentration. Lower temperature and longer reaction time favored xylose

and arabinose production. Optimum conditions were found to be 112 °C for 84.5 min. at 3.1% (w/v) H<sub>2</sub>SO<sub>4</sub> concentration<sup>97</sup>.

These optimum conditions also limit the production furfural from pentoses, an initiative of cellulosic ethanol. This is a potential issue for DDGS given its high hemicellulose content. Furfural production in acid hydrolysis of DDGS was found to be lower at 120° than at higher temperatures<sup>98</sup>. In the same study conversions of carbohydrates to monomeric sugars was found to be greatest, 37.2%, at 120 °C and 10% substrate loading (wt./vol.) for 60 min. This is equal to 20.5 mg sugar/mL liquor. Of which 3.7, 9.2, 1.5, and 6.1 mg/mL was glucose, xylose, galactose, and arabinose respectively.

## **2.4 The Advantages of Black Soldier Fly Bioprocessing of Plant Biomass**

The black soldier fly has unique characteristics that hold potential to contribute to the production of lignocellulosic ethanol. The black soldier fly (BSF), *Hermetia illucens*, (Diptera: Stratiomyidae) is a large (13-20mm) wasp-like fly<sup>99</sup>. The feeding activity of the BSF occurs only during the larval stage, and therefore it is not considered a disease vector or pest organism<sup>100</sup>. The larvae are saprophagous, and can be found in decomposing materials such as rotting fruit, animal carcasses, food waste, and notably manure<sup>101,102</sup>. The BSF is capable of digesting waste faster than any other known fly species due to powerful mouthparts and digestive enzymes<sup>103,104</sup>. Digestion of cellulosic biomass by the black soldier fly larvae (BSFL) reduces the degree of cellulose crystallinity. Plant cell walls, where most lignocellulose is contained, are degraded by BSF digestion, enhancing the surface area of the cellulose, hemicellulose, and lignin to enzymatic hydrolysis. Greater sugar yields are subsequently realized compared to undigested plant biomass<sup>105</sup>. Current literature surrounding the enzymatic systems of the BSFL and its symbiotic microbiota is sparse however.

## **2.5 BSFL as a Novel Protein Source and Addressing Food Security**

In addition to the energy crisis outlined above, the agricultural industry is coming up against a protein shortage. Much of the protein that we feed to commercially farmed animals is sourced from fish meal. Fish meal is a commercial product made of fish, fish bones, and fish offal. It is usually cooked and ground, and may be pressed to extract excess fat. Due to a steady decline in catches of wild fish, and an increase in demands for fishmeal and fish oil for livestock

and aquaculture feeds, the price of both fish meal and fish oil have significantly increased<sup>106</sup>. This increases the total cost of livestock production.

BSFL have a comparable amino acid profile to fish meal<sup>107</sup> and can be raised in a sustainable manner through the bioconversion of low value commodities and agricultural waste products. In feeding trials, BSFL meal was found to be a suitable replacement for fish meal in diets fed to quail (*Coturnix coturnix japonica*) up to 50% replacement as determined by weight gain and feeding preference<sup>107</sup>. The high fat content of whole BSFL meal was found to increase satiety and reduce feed intake among quail. This supports the use of de-fatted BSFL meal, in order to maintain a high fat intake and weight gain. This approach also allows for commercial application of larval grease such as biodiesel production. The fat content of BSFL is dependent upon the feed source and is highly variable ranging from 15-50%<sup>108</sup>. This is an environmentally friendly approach to the protein shortage, and represents significant cost saving in the industrial production of farmed meat, particularly aquaculture and chickens. As the cost of fish meal increases, and the demand for meat increases due to the advances in living standards in developing nations, further cost savings can be achieved. As research shows, BSFL have the ability to increase the amount of ethanol which can be produced from lignocellulosic feedstocks, while concurrently generating a high protein high fat larval biomass.

Economic advantages of this system include the creation of multiple product streams from the bioprocessing of low-value lignocellulosic feedstocks such as dried distiller's grains with solubles (DDGS) into: a larval protein meal with superior amino acid and mineral profiles than DDGS; a larval fat or grease which can be turned into a high value biodiesel or incorporated into animal feeds<sup>109</sup>; ethanol generated from liberation of sugars from DDGS which remains after larval digestion; and a soil amendment from the sugar liberated DDGS.

Both BSFL and DDGS are used as high protein animal feeds. BSFL protein has a superior amino acid profile and higher lysine content compared to DDGS protein<sup>94,110</sup>. The high heat used in the drying step of DDGS destroys some of the heat labile lysine<sup>94</sup>. Given that it is an essential amino acid, and is often in short supply in traditional animal feeds, it has to be supplemented into the feeds. The higher lysine content of BSFL would also reduce the cost associated with lysine supplementation in animal feeds.

BSFL are incapable of de novo synthesis of ten essential amino acids lysine, tryptophan, histidine, phenylalanine, leucine, isoleucine, methionine, valine, arginine, and threonine<sup>111</sup>.

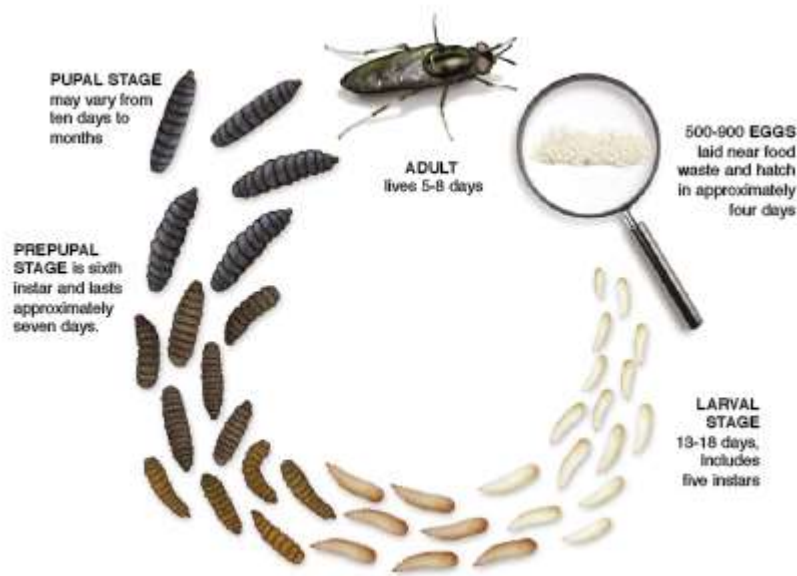
Therefore, they require lysine in their diet. Although the extent to which is unknown, the gut microflora of host organisms contributes to host amino acid through de novo synthesis of both fatty acid precursors and amino acids, and that microbial derived amino acids were incorporated into host proteins<sup>112</sup>.

Several groups have determined, through radio labeling of <sup>15</sup>N in <sup>15</sup>NH<sub>4</sub>Cl dietary supplementation, that microbial derived lysine represented a significant portion of the free plasma lysine pool in adult humans on nitrogen adequate diets. This was also shown in pig and rat models. These organisms cannot incorporate <sup>15</sup>N into essential amino acids, indicating a microbial origin. The extent to which de novo essential amino acid synthesis contributes to overall amino acid availability in host organisms is yet undetermined due to complications of <sup>15</sup>N method due to nitrogen recycling into and from the gut. Therefore lysine content in BSFL originates from dietary consumption through lysine in feeds and production by the gut microflora.

The use of feedstock pretreatments, such as solid state fermentation (SSF) serves to increase the nutrient availability of DDGS. This increases product yields, and further commercializes both DDGS and BSFL product usage. The use of flies and other insects in the bioprocessing of agricultural commodities has already spurred the creation of a cottage industry in the last few years. There are currently legislative and regulatory snags, but the use of insect proteins in the agriculture industry seems to be inevitable with the potential to revitalize our food supply chain, generate huge profits through new product generation and inherent cost savings, and increase the sustainability of the food system, particularly for systems involving fishmeal.

## **2.6 Rearing and Life Cycle Optimization of the Black Soldier Fly**

The lifecycle characteristics of the BSF as reported by Jong-Gill *et al.*<sup>101</sup> are as follows (Fig. 2-3). The egg, weighing 24 µg, is oval shaped with average dimensions of 887 µm along the longer axis, and 190 µm along the shorter axis. Females lay 1000 eggs on average which hatch in 81 hours at 27°C and 60% relative humidity. The larval stage lasts 15-20 days and depends on the larvae's ability to uptake nutrients from its surroundings. The cuticle of the prepupae slowly turns reddish-brown and upon pupation, the size decreases to 19 mm from the 21 mm larval size along the longest axis. This pupal stage lasts around 15 days. The adults are 13-20 mm long and are dark black in color. The adult lifespan can range from 5 days to 18 days.



**Fig. 2-3 Life Cycle of the Black Soldier Fly**

(Reprinted from: Unknown. Online. 7/08/2015. <http://www.staquaponics.com/#!/Black-Soldier-Flies-as-Fish-Feed/c1sbz/64A5A10E-D4F8-4632-88B5-9A07C933ADE9>).

Mating begins as early as the day preceding eclosion, and is the most frequent around 4-6 days preceding eclosion.

The larval stage of the BSF develops through five instars <sup>113</sup>, which is defined as a developmental stage of arthropods, such as insects, between each moult (ecdysis), until sexual maturity is reached <sup>114</sup>. The larval exoskeleton/cuticle contains chitin (a homopolymer of N-acetylglucosamine, a derivative of glucose), and is hardened through the addition of calcium carbonate which is known to be excreted through the larval integument of the BSFL in large quantities. This chitin is embedded in a protein matrix. In the BSFL the segments of the body are covered in rigid cuticle plates which form a true exoskeleton linked to adjacent plates by flexible membranes. The cuticles are hard and dark in color. They are said to be tanned, or sclerotized, and in the case of BSFL are also mineralized with calcium carbonate.

The BSF produces three generations in the southern United States and is active from late spring through early fall<sup>115</sup>. In the tropics, breeding occurs throughout the entire year<sup>102</sup>. These insects are from regions containing high amounts of natural sunlight throughout the year, and light has been shown to play an important role in mating and ovipositioning. Based on difficulty to stimulate mating in captive populations under artificial light, it was suggested that the eyes of

the male BSF may cue in to specific wavelengths of sunlight<sup>116</sup>. It was determined that wavelengths from 450-700 nm were responsible for influencing mating behaviors in the BSF<sup>117</sup>. This allows for the complete life-cycle of the BSF to be realized under laboratory conditions enhancing the research potential of this insect. In practice, many researchers tend to provide natural lighting to adult BSFs in order to maximize the number of offspring and to closely approximate their natural environments<sup>103</sup>.

Tingle *et al.* reported that mating occurs when males responded to “calling” females, and mating occurs with the pair positioned on the ground facing opposite directions while in coitus<sup>118</sup>. This runs contradictory to the earlier findings of Copello who identified that mating occurs in the air for this species<sup>119</sup>. Research by Tomberlin *et al.* elucidated the mating behavior. Males were observed to rest upon a leaf, and when a different male flew overhead or rested on the same leaf, the two males would meet in the air and grapple plummeting back toward the leaf. When they were 0.5-1.5 m above the leaf, the pair would separate with the victor returning to the leaf, and the other male vacating the area. Approaching females were treated in a similar manner, but during the grapple the pair would begin to mate<sup>120</sup>. A similar mating behavior has been noted for another Stratiomyid species *Hermetia comstocki*, and is defined as lekking or territorial behavior<sup>121</sup>. While the time of day and light intensity are significantly correlated with mating, temperature and humidity are significantly correlated with oviposition<sup>122</sup>.

The moisture content of the substrate has an effect on female oviposition as they will preferentially oviposit on substrates with optimal environments for the resultant larvae. The BSF prefers to lay its eggs onto the dry and cracked surface of moist substrates such as manure<sup>123</sup>. Fatchurochim *et al.*<sup>123</sup> reported that the BSF will oviposit onto substrates at moisture contents of 20-70%, but not higher. Most of the eggs were laid onto 40-60% moisture substrates. However, adult dry weight and head width were greater, and days to adult emergence were shorter for the eggs laid on 70% moisture substrates. This fact has been capitalized upon to develop egg collection strategies in the rearing of the BSF. In these methods, dry cardboard is suspended above optimal larval feed which has been saturated with water encouraging oviposition into the flutes of the cardboard for easy collection<sup>103</sup>. Bacteria also help mediate oviposition by producing a volatile chemical mixture which signals appropriate sites, as well as the presence of competitor species. A mixture of bacteria isolated from conspecific eggs was shown to be more effective at inducing oviposition compared to a single bacterial species<sup>124</sup>.

Temperature has a significant effect on the development and life cycle characteristics of the BSF as studied by Sheppard *et al.*<sup>115</sup>. They develop optimally at 27-30°C, and this is consistent with their identity as a tropical and warm-season temperate species. As the temperature increases smaller adults, shorter life spans, and lower survivorship are seen. While larvae held at 36°C had a high probability of becoming pre-pupae, less than 1% pupated. This may be due to an inability of the larvae to gain a sufficient amount of weight and energy during the larval stage. The authors proposed that the situation was similar to the larvae of the blowfly *Chrysomya rufifacies* (Macquart), (Diptera: Calliphoridae) which produces smaller larvae and adults at increased temperatures due to an increased metabolic rate and subsequent starvation<sup>125</sup>.

## **2.7 Diet of the Black Soldier Fly**

Early entomological study of the BSFL revealed that it sequesters calcium and produces calcium carbonate which is secreted through the hypodermis and covers the larval integument<sup>126</sup>. Sheppard *et al.* studied the effect of artificial diets on the life history and characteristics of the BSF, and found that when reared upon artificial diets, as is common in laboratory populations, the resulting adults are underdeveloped compared to wild BSFs<sup>103</sup>. Additionally, the final larval weight and pre-pupal calorie count per mg are lower in captive populations compared to wild BSFs, with the wild specimens having approximately 3.5 times as many calories.

It is yet unclear as to the extent that larval diet affects the life history traits of the BSF, since in the above study, the captive larvae were held in constant contact with their own waste which may exhibit toxicity. Optimal growth rates are established for BSFL when they are provided with as much fresh feed daily as they can assimilate in 4-6 hours<sup>103</sup>. The optimal rate of BSFL feeding has been determined for their use in so called CORS (Conversion of Organic Refuse by Saprophages) systems by Diener *et al.*<sup>127</sup>. A daily feed rate of 100 mg of chicken feed per larva was determined as the optimum rate for this system, and was a tradeoff between the reduction in organic matter and the larval biomass production (measured as the increase in pre-pupal dry weight). The feed was considered digested after it had undergone one pass through the BSFL's digestive system which resulted in a weight reduction of 41.8% (S.E. 0.61) in the feed. This feeding rate is also the threshold for development time as higher feeding rates did not significantly reduce the development time. At higher feed rates, the body mass of the adult is greater, but survivorship to the pre-pupal stage (but not adult stage) is reduced. The reduction in



dry matter, phosphorous, and nitrogen content of manure digested by BSFL was significantly lower among high feeding rates compared to lower ones<sup>128</sup>. The reduction in phosphorous and nitrogen contents of manure treated by BSFL digestion has been suggested to help reduce levels in the water supply due to field runoff, or leaching from lagoons thereby improving the water quality<sup>128</sup>.

The composition of the larval feed has an impact on the life cycle length of the BSF. When fed on a meat diet, the larval and pre-pupal stages were significantly longer than on a hen-feed diet. The larval and pupal mortality was also substantially lower for the hen-feed fed larvae, and the resulting adults were of larger size and better development<sup>129</sup>. This could be due to the elevated fat content present in the meat diet. Although fat is an essential dietary component for larvae, too much can retard their development as the larvae have a hard time metabolizing too much fat during metamorphosis into adults<sup>130</sup>.

The better development could also be due to the carriage of bacteria on conspecific eggs which are well suited for digestion of plant matter, and a smaller presence of bacteria which can efficiently digest meat. A review of the symbiotic bacteria associated with the BSF is given here in later section. When poultry manure was inoculated with *Bacillus subtilis* strains isolated from BSFL prior to addition of BSFL into the manure, the resulting larvae had 30% greater weight and a 10% reduction in development time compared to eggs placed in non-inoculated poultry manure<sup>131</sup>. This agrees with findings by Ye *et al.* which showed that bacteria isolated from the BSFL can be used as a probiotic for other BSFL colonies to enhance reduction in manure dry matter and larval development. Larvae are able to develop to pre-pupae and adults faster, although their longevity is not impacted by probiotic inoculation of feed. The use of probiotic feed results in adults of larger size<sup>131</sup>. Among the probiotic species was *Bacillus natto* which has been shown to have an antagonistic effect towards *E. coli* and *S. aureus*, as well as the ability to digest hemicellulose, pectin, and phytate<sup>132</sup>. Phytate is a major storage form of phosphorous in plants, especially brans and seeds. It is indigestible to non-ruminant animals as they lack the enzyme phytase. Phytates chelate minerals such as iron and zinc, preventing their uptake into the body<sup>133</sup>. Providing older or anaerobic feed to larvae is lethal or detrimental to their development due to the accumulation of toxic waste products, and a depletion of nutrients either by the larvae, or by microorganisms<sup>134</sup>.

The pre-pupae of the BSF can easily be self-harvested by directing their search for pupation sites into collection bins<sup>115</sup>. The type and presence of a pupation substrate has been shown by Holmes *et al.*<sup>135</sup> to have a significant effect on the pupation and adult emergence of BSFL. The BSFL grow optimally in moist substrates, but tend to vacate the moist larval feed and burrow into soil during the pre-pupal stage in order to protect them from predation and desiccation during pupation. Larvae not provided with a pupation substrate take longer to pupate than those with a substrate, and this is likely accounted for by excess time spent searching for suitable sites to bury themselves. These larvae also aggregated together, perhaps in an attempt to bury within the larval mass. Surprisingly, without a pupation substrate the larvae emerged faster than those with substrate, which may be an attempt to avoid desiccation or predation in the lack of cover. The survivorship to adult was much lower for those not provided a pupation substrate, suggesting that those larvae used up their energy reserves searching for pupation sites, and lacked the energy to complete metamorphosis. The packing density of the pupation substrate was inversely correlated with larval pupation time. Potting soil was the best among the substrates tested due to its ability to retain moisture, and its comparably low packing density compared to other substrates such as sand. Survivorship is generally lower in captive populations than in wild ones<sup>103</sup>. While almost all larvae survived to pre-pupae in captive populations, only around 25% emerged to adults compared to around 90% for wild populations. The wild survivorship to pre-pupae was not studied and no difference in the sex ratio among different populations was observed with all being around 60% female. In all populations, the female adult weight was greater than the males. The lifespan of the adult was greater in wild populations compared to captive ones.

## **2.8 Digestive Enzymes Native to the BSF**

Research done by Kim *et al.*<sup>136</sup> on the biochemical characterization of the BSFL's digestive enzymes has provided valuable insight into this topic which has largely been unreported, but is essential to understanding the mechanisms of bioprocessing by the BSF. Among 19 enzymes, which were screened for presence and activity level in the gut and salivary gland of the BSF, all were identified in the gut, and many of them were found in the salivary gland as well (**Table 2-2**). However, the salivary gland accounts for less than 10% of the total enzyme activity in the BSFL's digestive tract. This indicates that most of the digestion of feed

occurs in the gut of the insect. When compared to the common housefly, *Musca domestica*, all of the BSFL's gut enzymes showed at least identical enzyme activity to the housefly's respective gut enzymes. Leucine arylamidase,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase all exhibited greater activity in the BSFL's gut than in the housefly's gut. This confirms that the BSF has one of the most powerful digestive systems among fly species. The presence of lipase, indicative of zoophagous insects, and amylase, indicative of phytophagous insects, confirms the BSF as polyphagous insect. This polyphagous nature combined with high enzyme activity and diversity, shows that the BSF is a suitable insect for digesting a wide variety of waste and biomass.

**Table 2-2 Comparison of Enzyme Activity in Salivary Gland and Gut Extracts from *Hermetia illucens* and *Musca domestica* using Api-ZYM Kit, (From Kim *et al.* 2011)<sup>136</sup>.**

Enzymes	<i>Hermetia illucens</i>		<i>Musca domestica</i>	
	Salivary	Gut	Salivary	Gut
Control	0	0	0	0
Alkaline phosphatase	1	5	1	5
Esterase (C4)	3	3	3	3
Esterase lipase	3	5	3	5
Lipase	1	5	0	5
Leucine arylamidase	5 <sup>a</sup>	5	2	5
Valine arylamidase	0	5	0	5
Cystine arylamidase	0	5	0	5
Trypsin	0	4	1	4
$\alpha$ -Chymotrypsin	0	1	0	1
Acid phosphatase	4	5	3	5
Naphthol-AS-BI-phosphohydrolase	2	5	2	5
$\alpha$ -Galactosidase	0	4 <sup>a</sup>	0	1
$\beta$ -Galactosidase	3 <sup>a</sup>	5	0	5
$\beta$ -Glucuronidase	0	1	0	1
$\alpha$ -Glucosidase	3	5	2	5
$\beta$ -Glucosidase	0	1	1	1
N-Acetyl- $\beta$ -glucoaminidase	2	5	2	5
$\alpha$ -Mannosidase	0	4 <sup>a</sup>	0	1
$\alpha$ -Fucosidase	3 <sup>a</sup>	3 <sup>a</sup>	0	1

<sup>a</sup> indicates enzymes with more activity in *Hermetia illucens* extracts compared with those of *Musca domestica*.

Reprinted from Kim *et al.* 2011<sup>136</sup>, with permission from Elsevier.

It remains unclear to what extent the microorganisms present in the gut of the BSFL are responsible for the enzyme production and overall digestive process of cellulosic materials as literature in this area for this insect is sparse. However, the relationship has been extensively studied in termite systems<sup>137</sup>. Hindgut flagellate protists have long been known to be sources of cellulases and hemicellulases in the hindgut of lower termites<sup>138</sup>. In studies of protist

endosymbionts in lower termites, a large number of lignocellulosic genes in the glycosyl hydrolase family (GHF) were detected suggesting that these glycosyl hydrolases are involved in the protist's cellulose breakdown system. The major enzyme for the degradation of crystalline cellulose by these protists was found to be GHF 7 cellobiohydrolase. All of the cellulases in this study lacked the cellulose binding module (CBM), which is found in most microbial extracellular cellulases. CBM increases the concentration of cellulose at the surface of the cellulose. In the symbiotic protists, the cellulose is endocytosed and hydrolyzed within the organisms, and sufficient enzyme concentrations are capable of realization without CBM<sup>139</sup>.

## 2.9 Bacterial Diversity in the BSF

In humans, the number of microbes inhabiting the gut (around  $10^{14}$ ) outnumbers the somatic and germ cells of the human body (around  $10^{13}$ )<sup>140</sup>, and this community possesses metabolic activity equaling the human liver<sup>141</sup>. This led Lederberg and McCray<sup>142</sup> to coin the term microbiome to refer to an organisms native microbiota. Many insects are also inhabited by a large number and diversity of bacteria<sup>102,143,144</sup>. The microbiome of most insects is not well defined, an exception of which is the termite<sup>71,137,138,145-148</sup>. Despite the large variation in the numbers of microorganisms inhabiting different insects, it is likely that most insects have microbial populations whose numbers outnumber that of the host<sup>149</sup>. It is difficult to determine which of these organisms are native to their host species, and which is simply a transient organisms picked up from the environment, or food.

In order to colonize the gut, a bacterial species must multiply in their particular niche at a rate which exceeds the rate of their removal from the gut. The matter is complicated as some species are only able to colonize the gut in the presence of other members of the microbiota<sup>150</sup>. Additionally, the microbiome is capable of rapidly shifting in response to changes in the insect diet through introduction of enzymes, and changes in the microbial population<sup>151,152</sup>.

Microorganisms possess functions missing in the insect metabolism, and help to overcome barriers to plant consumption<sup>153,154</sup>. Microorganisms are also capable of degrading and detoxifying plant allelochemicals including flavonoids, tannins, and alkaloids which are secondary metabolites produced by the plant to protect against herbivory<sup>153,155</sup>. Microbial degradation of plant based aromatic compounds has been demonstrated in the termite<sup>156</sup>. Although digestive enzymes in some insects are derived from intestinal microorganisms at least

partially, studies to define the contribution have been primarily ambiguous<sup>157</sup>. This microbiome has many symbiotic functions including taking large roles in host digestion, physiology, development, and immune system, as well as preventing colonization by pathogens helping to maintain the host's health<sup>148</sup>.

Attempts to study native microbiomes of insects has been undertaken to elucidate the community structure of microorganisms specific to individual host species, and also to find bacterial genes which encode for enzymes of interest such as proteases, lipases, cellulases, xylases, and pectinases. Insects are of significant interest due to their large role in the degradation and recycling of organic wastes<sup>137,138,148,158</sup>.

Current thinking on the evolutionary relatedness between organisms is based in large part on phylogenetic trees. These trees are constructed from observed differences among biological molecules which are conserved over time, notably the genes encoding for small ribosomal subunits (16S rDNA for eukaryotes and 18s rDNA for prokaryotes)<sup>159,160</sup>. Upon analysis of these trees, it is seen that cellulose hydrolyzing, or cellulolytic, ability is present in the genetic code of many genera dispersed in the *Bacteria* domain, and most genera in the fungal group of the *Eucarya* domain. No cellulolytic members of the *Archaea* domain have yet been identified. Among the *Bacteria* domain cellulolytic ability is concentrated in the predominantly aerobic order *Actinomycetales* (phylum *Actinobacteria*) and the anaerobic order *Clostridiales* (phylum *Firmicutes*). Fungal cellulolytic ability is ubiquitous, being seen throughout the entire kingdom<sup>38</sup>.

Among the bacteria, cellulose utilizers can be classified into several broad categories: (1) fermentative anaerobes, mostly gram positive (*Clostridium*, *Ruminococcus*, and *Caldicellulosiruptor*), but with some gram negative (*Butyrivibrio*, *Acetivibrio*, and *Fibrobacter*); (2) aerobic gram positive bacteria (*Cellulomonas*, and *Thermobifida*); and (3) aerobic gliding bacteria (*Cytophaga*, and *Sporocytophaga*). Despite having the genetic information for cellulose utilization, only a few species are actively cellulolytic among those genera<sup>38</sup>. With few exception<sup>161,162</sup>, anaerobic bacteria degrade cellulose through a so called complexed cellulase system as exemplified by the highly characterized organelles of *Clostridium thermocellum*<sup>163</sup>. This species produces both extracellular and cell surface bound cellulases, but some anaerobic bacteria only produce cell bound cellulases, at least in measurable amounts. This may explain the necessity of anaerobes to be bound to the cellulose substrate for optimal growth.

The relatively new technique of pyrosequencing, an extension of the polymerase chain reaction (PCR), has shed new light on the nature and role of the microbiome of the BSFL. Pyrosequencing of 16S rDNA of BSFL fed different diets revealed that the insect's microbiome is dependent upon the feed source. More nutrient diverse feed sources resulted in a greater diversity of gut bacteria<sup>164</sup>. Utilization of pyrosequencing, which is capable of sequencing so-called unculturable bacteria, is still presented with the problem of differentiation between autochthonous and allochthonous species<sup>164</sup>. It has also been shown that the microbiome shifts with each life cycle<sup>165</sup>. In a study by Jeon *et al.*<sup>164</sup>, four aerobic bacteria were isolated from the BSFL's gut and cultured, having been selected for their high ability to degrade organic compounds. These organisms were identified via 16S rDNA sequencing and were found to be the following: (1) *Bacillus amyloliquefacien ssp.* which showed high protease, amylase, and cellulase activity; (2,3) *Bacillus stratosphericus sp.* and Family *Paenibacillaceae sp.* which showed high lipase and cellulose activity; and (4) *Paenibacillaceae mirabilis sp.* which showed high protease and lipase activity<sup>164</sup>.

In another study by researchers Zheng *et al.*<sup>124</sup> bacterial species were identified across different life cycle stages of the BSFL. In general, the bacterial diversity was found to be similar across life stages of the BSF at the phylum level, but at the genus level diversity was higher for larvae, pre-pupae, pupae, and adult than for the egg due to the sessile nature of the egg<sup>165</sup>. Yu *et al.*<sup>166</sup> isolated 5 bacterial strains from the integument and 5 from the gut of the BSFL. They were all identified to be *Bacillus subtilis* by 16S rDNA sequencing to determine the gens and PCR of the DNA gyrase B protein encoding genes to determine the species. The *Bacillus* genus is composed of gram positive, rod-shaped bacteria belonging to the phylum Firmicutes. Each of the isolated strains was identified as being unique, and it was determined that they have the ability to digest casein, gluten, and organic phosphorous.

In addition to bacterial symbiosis, some insect have a symbiotic with fungal organisms such as leaf-cutting ants of Central and South America such as *Atta sexdens*, and mound-building termites of Africa and Asia such as *Macrotermes bellicosus*. These insects use cellulose rich plant material to cultivate fungal monocultures which are the food source for the mycophagous insects. *Laboulbeniomycetes* and *Septobasidium* are groups of fungi that can be found on arthropods particularly the former. Individual colonization of an insect body by these fungi is

generally considered an infection of the host. Flies are particularly susceptible to entomophagous fungi, which can endanger an adult colony<sup>167</sup>.

## **2.10 Manure and Waste Management with the BSFL**

The BSF is a filth fly, and is commonly found in places containing high concentrations of animal manure. It was observed that the housefly populations in confined animal feeding operations colonized by the BSF were significantly lower than those where the BSF was absent<sup>100,113,118,168</sup>. Reduction in housefly populations have been reported between 94-100%<sup>100</sup>. BSFL prevent ovipositing by houseflies depends upon the time that the larvae have been in the feed, and 4-5 days of BSFL larvae occupation in the feed was determined sufficient to discourage housefly populations<sup>100</sup>. Wild houseflies are 10 to 30 times more resistant to the insecticides cyromazine and pyriproxifen than BSFL<sup>169</sup>. This may be due to behavioral differences as most wild adult BSF are not present around animal confinement facilities where the insecticides are used, limiting their exposure. The houseflies, which maintain a presence in order to feed as adults unlike the BSF, have a greater exposure and therefore more selective pressure to develop resistance to insecticides. The BSFL aerate and dry manure reducing odors by the consumption and digestion of microorganisms, as well as by producing bacteriostatic, bactericidal, and fungicidal compounds<sup>100,170-172</sup>. Tingle<sup>118</sup> showed that BSFL reduce waste in poultry facilities, and Sheppard<sup>100</sup> showed that the reduction in manure accumulation was between 42-56%. The BSFL also reduced the manure's nitrogen concentration and other nutrients which reduces the potential for pollution.

Aside from pollutant and manure dry matter reduction, the dried pre-pupae can be used directly as feed for a variety of livestock including poultry, swine, and aquaculture as they contain 42% protein, and 35% fat<sup>110,115,127</sup>. The dried pre-pupae feedstock has an estimated value equaling soybean, or meat and bone meal. This value can be increased if they are used live, as specialty feed, or marketed to exploit some of the unique attributes (chitin, or fatty acid composition) of the insect. The optimal yield of feedstuff to manure on a dry weight basis was found to be 7.8%<sup>115</sup>. The pre-pupae cuticle or exoskeleton contains the polysaccharide chitin which has been shown to reduce the digestibility of the BSF, and prevents uptake of other nutrients when used in some aquaculture systems<sup>127,173</sup>. When used as a fish meal replacement at

less than or equal to 33%, the BSF pre-pupae were a suitable replacement showing good growth gains in juvenile turbot fish<sup>174</sup>.

In addition to manure, BSFL have been successfully used to treat leachate from food waste compost. From one week of growth on compost leachate where the decaying vegetable matter was replaced twice weekly, the larval mass increased 70.7% on average. The larvae were able to significantly reduce the chemical oxygen demand and increase the pH, while reducing the concentration of volatile organic acids (VOAs) and alcohols, and clearing the leachate of ninhydrin positive metabolites. Ninhydrin is a chemical used for detection of ammonia, or primary and secondary amines. Compared to controls without BSFL, a seven to nine-fold reduction in acetic, propanoic, and butyric acid was seen over two days. In a comparison of sterile and non-sterile leachates, the VOAs and alcohols were reduced by up to one-half the rate seen when BSFL were included in the leachate. Larval processing of the leachate increased the ammonium concentration approximately six fold over seven days<sup>175</sup>. BSFL significantly increased the nitrogen-mineralization by increasing the concentration of ammonia. They also facilitate the recovery of  $\text{NO}_3^-$  in compost leachate fractions via dissimilatory nitrate reduction to ammonia pathway<sup>176</sup>. Most insects store nitrogen waste as uric acid which primarily precipitates in the insect's rectum<sup>177,178</sup>. Researchers have identified allantoin, the immediate degradation product of uric acid, and a water-soluble waste product, in waste excreted by Dipterans, as well as  $\text{NH}_4^+$  albeit to a lesser extent<sup>177,179,180</sup>.

## 2.11 Anti-Microbial and Anti-Parasite Actions of BSFL

BSFL produce fungicidal compounds which support the immune system of the fly as it feeds on decaying matter<sup>164</sup>, and unlike houseflies, BSFL can reduce *E. coli* populations in feces<sup>172</sup>. They have been shown to reduce other pathogen populations in manure treatment systems also<sup>170,172</sup>. This antimicrobial action helps the BSFL to resist infection from pathogenic organisms, as well to digest the food for the larvae by colonizing the food with symbiotic bacteria which facilitate the breakdown of macronutrients<sup>149</sup>. For this reason so called maggot treatment has been successfully used medically to treat infected wounds, chronic or necrotic infections, osteomyelitis, and burn wounds<sup>181-185</sup>.

Methanol extracts of BSFL inhibited the growth of *Klebsiella pneumonia*, *Neisseria gonorrhoeae*, and *Shigella sonnei* which are all gram negative. The extracts did not exhibit



antagonistic effects towards gram positive species such as *Bacillus subtilis*, *Streptococcus mutans*, and *Sarcina lutea*<sup>186</sup>. BSFL were not able to destroy or inactivate the eggs of the intestinal parasite *Ascaris suum*, which is found in the intestine and manure of infected swine<sup>187</sup>.

Hexanedioic acid isolated from BSFL extracts was found to have antimicrobial activity against *Klebsiella pneumoniae* in vitro, and in vivo using mice infected with *K. pneumoniae* at levels comparable to commercial antibiotics<sup>188</sup>. Hexanedioic acid exhibited anti-microbial effects against gram-negative bacteria, and was found to be safe in long term toxicity studies using mice, rats, rabbits, and hamsters at high doses for 33 weeks. This indicates that it may have potential for use as a novel antibiotic. Black soldier flies are still susceptible to infection however.

BSF eggs and younger BSF larvae are more susceptible than older life stages to fungal infection and death<sup>103</sup>. This fact has been exploited as a biological control measure to reduce fly populations in poultry operations<sup>113</sup>. Some evidence suggests that bacteria associated with BSF eggs helps to protect against fungal infection<sup>189</sup>. It is reasonable to assume that enzyme activity may be imparted to the BSFL through digested fungal enzymes, but BSFL are not reported to have a fungal gut community in healthy populations. An advanced understanding of the microbiome of the BSFL and its functions are essential to enhance its utilization in commercial applications, of which historical importance has been use of the larvae in manure management systems.

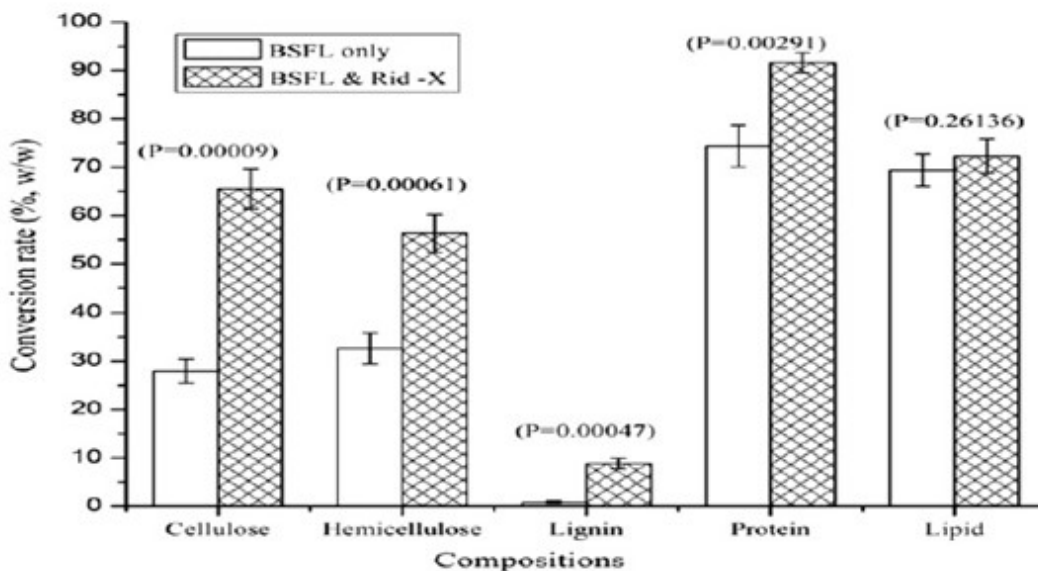
## **2.12 Biodiesel Production from BSFL**

Similarly to lignocellulosic ethanol, biodiesel production relies heavily on food resources, mainly crop oils, and alternative feedstocks are being continuously evaluated. In addition to collecting pre-pupae for commercialization as a livestock feed, BSFL grown on pig, chicken, and cattle manure can be used for the production of biodiesel<sup>105</sup>. Li *et al.*<sup>190</sup> utilized BSFL for the reduction in dry matter of dairy manure from various animals which resulted in the production of a high protein and high fat dried larval biomass. Soxhlet extraction with petroleum ether was utilized to obtain BSFL grease (purified with 0.5% H<sub>2</sub>SO<sub>4</sub>). A 2-step biodiesel production process, including pretreatment with 1% w/w H<sub>2</sub>SO<sub>4</sub> as the catalyst, and transesterification with 0.8% w/w NaOH as the catalyst, both at 8:1 methanol/oil ratio, was used. Fermentable sugars

were obtained from the digested manure via pretreatment with dilute 2% HCl and subsequent cellulose enzyme hydrolysis.

This novel system not only reduced the dry matter of manure, a potential source of environmental pollution due to large amounts of undigested organic matter<sup>191</sup>, but converted it into larval biomass capable of being produced into biodiesel, and enhanced the recovery of fermentable sugars which can be used for ethanol production. Feeding the larvae chicken manure resulted in the highest biodiesel yield compared to cattle manure and pig manure fed larvae. The nutritional composition of chicken manure allowed for the greatest gains in insect biomass during the growth period. The resulting biodiesel had comparable properties to rapeseed oil biodiesel, and met the European standard EN14214<sup>190</sup>.

In another study conducted by researchers Zheng *et al.*<sup>192</sup> BSFL were fed residual solid fraction of restaurant waste (SRF) following biodiesel production from the restaurant waste. The results are shown on **Fig. 2-4**. Biodiesel was made from the larval grease in a modified version of the method used by Li *et al.*<sup>190</sup> The fat content of the larvae was higher, but the fatty acid profile was very similar, suggesting both that: SRF was a better media for larval growth and development than manure; and that the fatty acid composition of the larval grease was independent of the larval feed material. The larvae were able to reduce the mass of the SRF by



**Fig. 2-4 Degradation Rate of Selected Components in the Mixed Medium of RSW (70%) and Rice Straw (30%) after Conversion by BSFL with or without Rid-X (0.35%)**

Reprinted from Li *et al.*<sup>192</sup>, with permission from Elsevier.

62% over a 7 day digestion period. The moisture content was reduced from 81% to 71% thereby increasing the percent solids. In a follow-up study, biodiesel was produced from the BSFL grease (also prepared via Soxhlet extraction of dried and ground larvae) resulting from larvae which were fed on rice straw and restaurant solid waste (RSW)<sup>109</sup>. In order to facilitate digestion of the feedstocks Rid-X was added to the larval feed. Rid-X, which is a commercial product containing microbes and enzymes, helped to hydrolyze the high cellulose feedstocks, and increased the final weight of the larval biomass. It was more difficult for the larvae to digest cellulose than other feedstock components evidenced by the fact that larval grease yield increased as the ratio of RSW to rice straw was increased<sup>192</sup>. When 0.35% w/w of Rid-X was added in the 1000 g mixed feed (RSW:Rice Straw, 7:3) 122.8 g of insect biomass was obtained, and BSFL grease content reached 39.6% of the larvae, yielding 43.8 g biodiesel from 2000 larvae<sup>192</sup> (**Fig. 2-4**).

### **2.13 Solid State Fermentation of DDGS**

Because BSFL produce low levels of cellulases, their application for use in highly fibrous feedstocks is limited<sup>136</sup>. They have shown the ability to reduce the crystallinity of cellulose through the micronization of cell walls, but may benefit from the addition of enzymatic pretreatment of lignocellulosic feedstocks<sup>192</sup>. A novel cellulase have been identified from metagenomics screening of the gut microflora of the BSF which possess a high temperature and pH stability range, as well as low activity losses from 10% polar organic solvents, 1% non-ionic detergents, and 0.5M denaturing agents. Thin layer chromatography suggests that it is an endo- $\beta$ -1,4- glucanase<sup>193</sup>. However, anecdotal evidence suggests that the BSFL avoid lignocellulosic materials in their diet. SSF is an attractive pretreatment to BSFL digestion of DDGS due to its ability to produce extracellular enzymes which can degrade the constituent matrices in DDGS.

Many species and strains of organisms have been used in fermentation, but filamentous fungi are the best organisms for SSF due to their low water activity requirements and ability to penetrate into and between substrate particles. Compared to submerged fermentation SSF has a lower capital cost, lower energy requirements, needs simpler fermentation substrate, and requires a less rigorous control of the fermentation parameters<sup>194</sup>. Wood rot fungi secrete much higher levels of extracellular enzymes than yeast or bacteria, and white-rot fungi in particular are able to degrade all plant cell wall components, including lignin<sup>194</sup>.

The most used white-rot fungi is *Phanerochaete chrysosporium*. Because of its high fiber and protein content, DDGS is a suitable substrate for SSF. The addition of urea to DDGS for SSF applications has been shown to increase the cellulase and particularly the xylanase production by filamentous fungi<sup>194</sup>. The porosity of DDGS is important to the growth of filamentous fungi, and tighter packing of DDGS suppresses growth and enzyme production<sup>194</sup>. Fungal organisms used in SSF applications can be used to concurrently and/or subsequently ferment DDGS which can have a superior effect over monoculture fermentations towards the production of enzymes and the resulting changes to the DDGS<sup>195</sup>. SSF of DDGS generally has the effect of reducing the fiber content and increasing the protein content of DDGS<sup>195</sup>.

*Aspergillus* stains have been used in SSF applications due to their ability to produce cellulolytic enzymes, as well as their perceived status as a safe organism. Citric acid and many enzymes produced by *Aspergillus niger* have obtained generally recognized as safe (GRAS) status by the United States Food and Drug Administration. *A. niger* has been exploited commercially for the production of many enzymes, most notably pectinase, protease, and amyloglucosidase. *Aspergillus* application in food systems raises concerns regarding toxin production such as aflatoxin. No known case of aflatoxin or trichotecenes production by *A. niger* has been proven. *A. niger* is capable of producing the nephrotoxic and carcinogenic mycotoxin ochratoxin A, although only 6% of *A. niger* isolates were shown to produce this toxin<sup>196</sup>.

Mycotoxins are a known contaminant of DDGS resulting from contaminated grains used in the dry-grind ethanol process. Like other constituents, they are concentrated during the manufacturing process. Minimum advisory levels of mycotoxins in DDGS for use in animal feed are provided by the United States Food and Drug Administration (U.S. FDA). In a survey of DDGS in 2010, it was found that mycotoxins in DDGS were below the detection limit in nearly every case, except for zearalenone which contained between 100 – 300 µg/kg<sup>197</sup>. Therefore mycotoxin concerns, while justified, are not likely to be a large issue. It is unknown yet how these toxins are impacted or concentrated through feeding to BSFL.

### **3 HYPOTHESES AND OBJECTIVES**

The overall hypothesis for this research is that the combination of SSF and BSFL digestion will increase the degradation of DDGS constituent matrices, particularly fiber, which will increase the recovery of fermentable sugars from DDGS. Under this overarching hypothesis, four items are individually hypothesized. The first hypothesis is that the concentration of enzymes in spent larval feed is dependent upon the composition of the starting feed. Since most enzymatic activity is found in the larval gut, the microbiome of the BSFL is most likely primarily responsible for enzyme production. The microbiome of the BSF has previously been shown to shift based on the available nutrients in their environment and thus on the larval feed source. The second hypothesis is that SSF using bacteria isolated from the BSF and cellulose-degrading fungi will improve the nutrient profile and digestibility of DDGS. The third hypothesis is that SSF utilizing bacterial and fungal isolates will increase the liberation of sugars via enzymatic hydrolysis from DDGS by opening the component matrices therein, including that of lignocellulose, which will increase the susceptibility of cellulose to enzymatic hydrolysis.. The fourth hypothesis is that fermentable sugar yield, produced by acid hydrolysis of DDGS digested by BSFL, will increase during the larval digestion period, and that the digested nutrients can be recovered in the form of a high fat, high protein larval biomass.

Objectives of this research are:

- 1) to test for general protease, lipase, amylase, and cellulase activity in a variety of spent larval feeds;
- 2) to determine feed consumption extent and rate as well as average larval weight gain;
- 3) to measure the decrease in cellulose crystallinity to evaluate the influence of cellulose structures;
- 4) to measure the change in fermentable sugars produced by both acid hydrolysis pretreatment and enzymatic hydrolysis both before and after digestion of feed stocks; and
- 5) to quantify the changes in DDGS during SSF and larval digestion via proximate analysis.

## 4 STUDY 1: CHARACTERIZATION OF ENZYMES FROM SPENT LARVAL FEED

### 4.1 Abstract

Secretion of enzymes from the BSFL into their feed bed can potentially contribute to the insects DDGS digestive capabilities along with symbiotic bacteria associated with the BSFL. The nutrient composition of the BSFL's diet may impact their digestive capabilities. To investigate this hypothesis, and specifically the effect of the fiber content of the feed, enzyme activities were first analyzed in feed beds which varied in cellulose content using food commodities (corn meal, DDGS, and whole milk powder) containing complex matrices to evaluate the effect of cellulose content directly. Enzyme activities in spent larval feeds were shown to decrease as the cellulose content of the feed increased. From these BSFL spent feeds, bacteria associated with BSFL were isolated and included *Lactobacillus plantarum*, *Sphingomonas melonis*, *Psuedomonas sp.*, and *Klebsiella pneumoniae*. These bacteria should have originated from the digestive system of the BSFL, which were hypothesized to play a role in the BSFL's digestive capabilities. The contribution of these isolates in enzyme activities were analyzed, and *Lactobacillus plantarum* showed the greatest overall enzyme activity based on protease, cellulase, amylase, and lipase assays of the isolated bacteria. Because the digestive capabilities of BSFL were shown to decrease as the cellulose content of their feed increases in this study, we further hypothesized that fermentation of cellulosic materials prior to BSFL digestion can assist the contribution of the larvae in glucose recovery from the cellulosic materials, which was investigated in Study 2.

### 4.2 Experimental Approach

#### 4.2.1 Materials

DDGS was kindly provided by NorthWest Bioenergy (Unity, SK). Corn meal and whole milk powder were purchased at grocery stores. The BSFL used in this study were purchased

from Supercricket and Bugs4Pets.com. All chemicals used in this study were commercially available ACS grade, and were purchased from VWR International (Edmonton, AB) and Fisher Scientific (Ottawa, ON). Amicon ultrafiltration Cell Model 8050 (Amicon Corp., Danvers, MS, USA) with Millipore Ultrafiltration Membrane YM10 (Millipore Corporation, Billerica, MA, USA) were used for protein concentration. The spectrophotometer used was a ThermoFisher Scientific, Model G10S UV-Vis (Madison, WI, USA). Protein concentrations were measured using the CB-X Protein Assay (G Biosciences, St. Louis, MO, USA). Extraction of bacterial DNA was done using the EZ-10 Spin Column Plasmid DNA Kit, and was purified after PCR using the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Markham, ON). The DNA sequencing was performed by the National Research Council Canada (Saskatoon, SK). Centrifugation was performed using DuPont Sorvall Instruments Model RC-5C Centrifuge. Microfuge centrifugation was performed using a Beckman Coulter Microfuge 16 Centrifuge (Indianapolis, IN, USA).

#### **4.2.2 Screening of Spent Larval Feeds for General Enzyme Activities**

Non-synthetic feed blends differing in cellulose content were made by blending corn-meal and wheat DDGS in ratios of 1:0, 1:1, and 0:1 respectively to 90% (dwb) of the total feed weight needed. 10% (dwb) whole milk powder was added to the feed blends as source of fats for complete larval nutrition. Crude protein extracts (CPE) were prepared by suspending 5g spent feed in 25 mL 50 mM Tris-HCl buffer with 100mM NaCl, pH 7.5, homogenized via vortexing, and fractionated using centrifugation at 4°C, 8000 rpm, for 20 min (Sorval SS-34 rotor). The supernatant was decanted, and the pellet was re-homogenized with 5 mL of extraction buffer. After centrifugation of this latter solution, the supernatant was decanted and combined with the first fraction. The CPEs were concentrated with the use of an Amicon membrane filtration unit with a 10 kDa cutoff limit to reduce the storage and working volume of the solution for assays. This cutoff size was chosen to retain the reported size for all enzymes of interest<sup>198-202</sup>. The concentrate obtained after filtration was preserved by the addition of an equivalent volume of glycerol and stored at -20°C for further use. Five CPEs were made for each synthetic feed blend.

The protein concentration was measured using the CBX-Protein Assay, which is based on the Bradford/Coomassie Brilliant Blue assay. The red form of the Coomassie dye donates an electron to the ionizable protein sites, opening the protein structure. The dye then binds to the

exposed hydrophobic protein regions through van der Waals forces, stabilizing the blue form which is read at 595nm on a spectrophotometer.

Protease enzyme activity was measured by spectrophotometric measurement of a standard casein solution at 280nm following enzyme exposure for 9 minutes at 25°C. At the end point of the assay the reaction mixture was acidified and large proteins and peptides were removed by centrifugation at 12,000 rpm for 5 minutes in air microcentrifugation. The supernatant was used for absorbance measurements of solubilized free peptides. A280 measures the absorbance of aromatic, hydrophobic amino acids tyrosine and tryptophan of liberated small soluble peptides from the casein interior<sup>198</sup>. Acidification of the casein solution facilitates precipitation of large proteins.

Cellulase activity was determined using microcrystalline cellulose as the substrate. After the treatment of cellulose with enzyme preparations in a 0.05 M acetic acid, pH 5.0 buffer solution, the amount of glucose liberated from cellulose was determined and the activity was calculated as the ability to liberate glucose in a unit of time. To this reaction mixture, 3,5-dinitrosalicylic acid (DNS) is added. DNS is reduced by the liberated reducing sugar to a darker colored compound which is measured spectrophotometrically at 540 nm, and from which the enzyme activity can be calculated based on moles of reducing sugar released<sup>202</sup>. The slope of a blank solution containing no enzymes, which showed the non-enzymatic release of glucose over time from microcrystalline cellulose in the acidic buffer, was subtracted from this slope, which was fed into the linear equation of the standard curve to obtain the concentration of reducing sugar liberated per minute. Two endpoints of 60 and 120 minutes were utilized to obtain a slope for the A540 per minute.

The amylase assay was performed in pH buffered solution of 0.02M sodium phosphate, pH 6.9 with 0.006M sodium chloride that contains 0.5 mg/mL of soluble starch. The enzyme preparation was allowed to act against this reaction mixture for a set time. The sugar in the supernatant of the reaction mixture was determined by DNS measurement as outlined above. Samples were measured at two 0, 3, and 9 minutes, and the enzyme activity was calculated from the slope of the absorbance over time.

A titrimetric method was used to calculate the lipase activity in units per g of spent feed<sup>200</sup>. The lipase activity assay measures the rate of hydrolysis of an olive oil emulsion determined by potentiometric titration using a pH meter and standardized NaOH. The emulsion was prepared by



first making a 10% gum arabic solution in water. To this solution, oil (20% of final) and crushed ice were added and blended at low speed for several minutes. The emulsion was filtered through glass wool to remove insoluble gum arabic and contaminants. The sample was added to a salt solution containing calcium and albumin, which helps to reversibly bind free fatty acids, and the pH was adjusted to 8. The moles of standardized NaOH used to maintain the pH at 8 over 10 minutes was equated to moles of free fatty acids liberated per minute which was then converted to units per gram of feed.

#### **4.2.3 Isolation of Potential Cellulolytic Symbionts from Spent Larval Feeds**

Spent larval feed composed of corn meal and whole milk powder were suspended in sterile H<sub>2</sub>O and serially diluted in sterile H<sub>2</sub>O, then plated onto MRS agar media plates in order to obtain an estimate of the number of viable, culturable cells existing in the spent larval feed. Dilute suspensions of spent larval feed were again plated on MRS media agar plates which were incubated at 37°C in aerobic and anaerobic conditions. Colonies possessing different oxygen requirements or colony morphologies were streaked onto MRS plates in order to obtain a pure bacterial isolates. These isolates were grown in liquid MRS broth, which was degassed for anaerobes, at their respective oxygen requirements, then preserved with the addition of glycerol to 50% and stored at -80°C. Through 16S rDNA sequencing the isolates were identified and considered for potential utilization in a feedstock pretreatment step during Study 2.

#### **4.2.4 Lignocellulose Feed Content Effect on Enzyme Activity**

Using the methods of Li et al.<sup>190</sup>, young (10d) larvae were to be washed and placed into different feed groups identical in weight. Due to difficulty sourcing eggs, and determining the age of the larvae, the larval age was widely distributed. The different feed groups were composed of feeds differing only in the concentration of cellulose to control for effects of differing feed nutrients on enzymatic activities of the larval digestive systems.

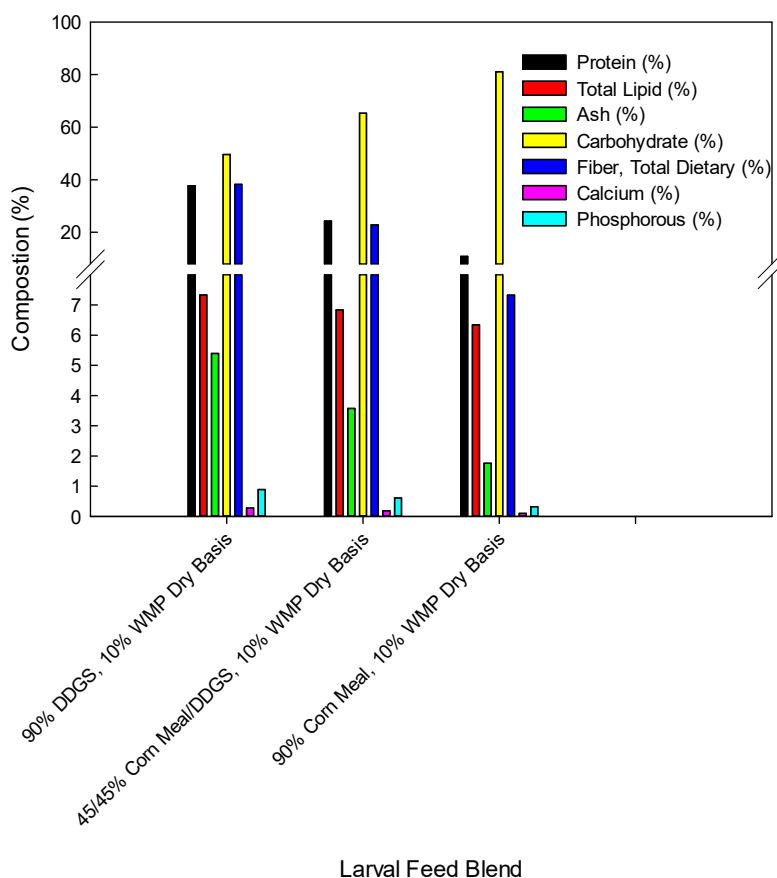
The lignocellulose content was adjusted by blending a 90% corn meal/10% whole milk powder mixture with cellulose in fractions of 0, 25, 50, 75, and 100% cellulose on a dry weight basis. The blended feed was mixed with sterile distilled water to a moisture content (DDGS starting moisture content was 4.4 ± 0.1%) of 60%, and then sterilized via autoclaving for 20 minutes at 121°C. Larvae were added to the feed in a 2:1 wt./wt. ratio (wet-weight basis). At

days 0, 2, 4, 6, and 8, a portion of the substrate and larvae were removed for sampling. The difference in enzyme activity present in spent feeds was measured as described in the previous section.

### 4.3 Results and Discussion

#### 4.3.1 Screening of Spent Larval Feeds for General Enzyme Activities

In this section, substrate which had been digested by BSFL was analyzed for enzyme activity. This was done to evaluate the effect of the feeding substrate, and specifically the fiber content of that substrate which is higher in DDGS than cornmeal, on the digestive abilities of BSFL. **Fig. 4-1** shows the composition of feed blends used in this study. Wheat DDGS has a high protein content of 38.9% (dwb), compared to corn meal and WMP which have 9.05 and

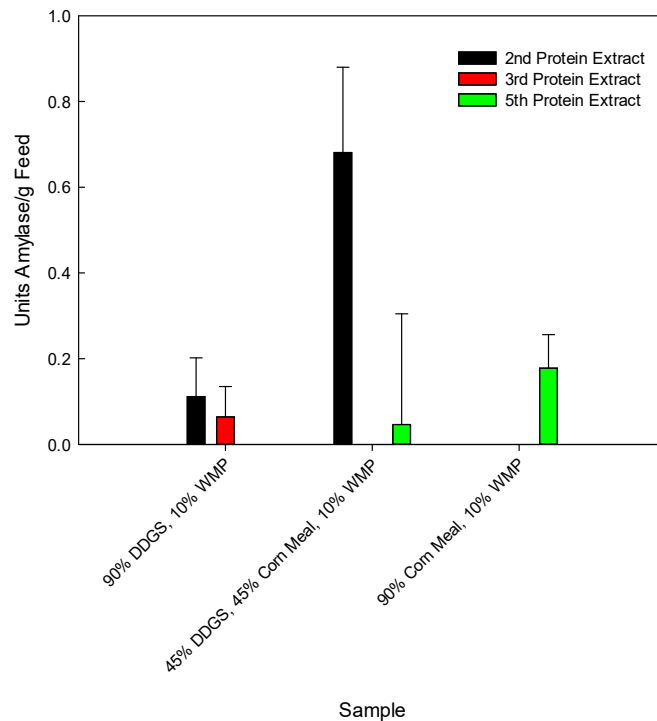


**Fig. 4-1 Proximate Analysis of Larval Feed Dry and Wet Basis**

N = between 3 and 39 for the reported parameters, and varies for each sample component and assay;

26.32% protein (dwb) respectively. Therefore feed blends containing more DDGS than cornmeal have a lower relative composition of carbohydrates, and higher protein content. The fiber content of DDGS is also much higher than that of cornmeal. Most of the fat content is derived from whole milk powder and is relatively consistent across all three feed blends, while ash content decreases as DDGS content decreases. This data was obtained from the USDA nutrient database resource for corn meal and WMP<sup>209,210</sup>; and from the University of Saskatchewan's wheat DDGS nutrient profile webpage<sup>211</sup>. It was compiled to model the feed blends used in this study, and the results are shown on a dry basis. Protein concentrations of the CPEs from spent larval feed were measured using the CBX-protein assay. The extracts were numbered in the order they were made, so higher numbers mean that the extracts were made at a later date during larval digestion of feeds. These extracts were used to measure the activities of enzymes secreted from the BSFL digestive system into their feed bed. The protein concentrations of the CPEs closely matched the relative protein concentrations of the substrates. DDGS, which has a much higher protein concentration than corn meal, resulted in higher protein concentration CPEs when used higher concentrations in the blends. Therefore the amount of protein in the CPE was correlated well to the amount of DDGS in the substrate for each extract. Concentration of the CPEs, using membrane filtration, served to increase the protein, and presumably the enzymes of interest, by ~100%. Originally, the protein concentration was monitored as a potential marker of the calculated enzyme activity in a sample (e.g., U/mg protein). The protein content of the sample does not necessarily correlate to the enzyme activity in the sample however. Since the enzymes present are presumed to come from excretions of the BSFL gut, and digestive action of the BSFL change the substrate composition, using protein concentration as a measure of potential enzyme amount present is not likely accurate. This method was changed instead to calculating enzyme activity as a function of the weight of digested feed represented in the CPE and their subsequent concentrates. While this method does not give an absolute value for the activities of the enzymes present in the sample, it does provide a more sensible value for comparison across samples.

As shown on **Fig. 4-2**, amylase activity was present in 90% DDGS extractions 2 and 3, 45% DDGS extractions 2 and 5, and 90% corn meal extraction 5. The 45% DDGS extraction showed the highest amount of amylase activity, and the lowest amount of variation among replicates as a percentage of the mean. All other samples showed similar, comparably lower amylase activity with high coefficients of variation.

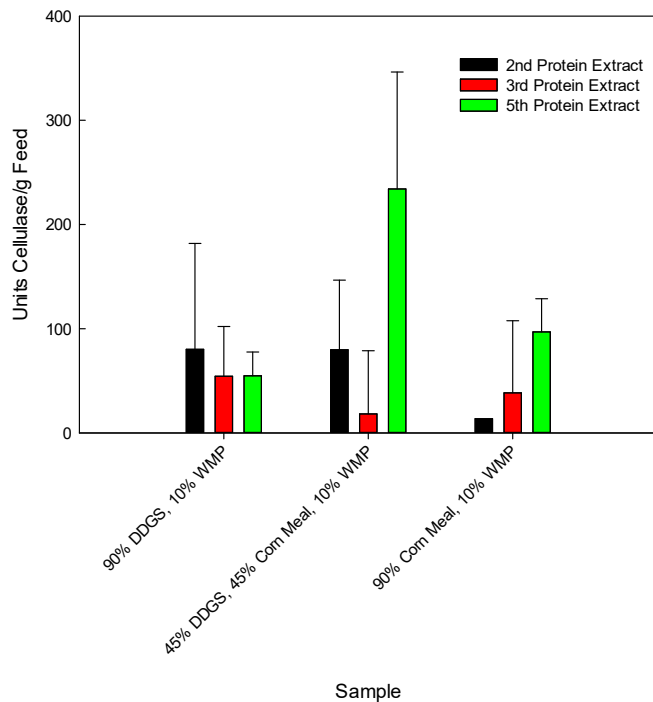


**Fig. 4-2 Alpha-Amylase Activity of Concentrated Crude Protein Extracts Preserved with Glycerol**

Feed blends were made of DDGS, cornmeal and whole milk powder. After BSFL digestion, the spent feeds were extracted with 50 mM Tris-HCl buffer with 100mM NaCl, pH 7.5. The extracts were numbered in the order they were made, so higher numbers mean that the extracts were made at a later date during larval digestion of feeds. N = 3; mean and std. dev.; Not significant at  $\alpha = 0.05$ ;  $p = 0.41$ ;

The cellulase contents of the protein extracts were measured at this time, and the results are shown in **Fig. 4-3**. Some cellulase activity was observed, but no trend across protein extracts or feed blend compositions was seen.

The results for the protease activities of the CPEs are summarized in **Fig. 4-4**. There was a lot of difficulty in obtaining statistically significant data for enzyme assays of the concentrated crude protein extracts. The problem may lie in the large number of freeze thaw cycles which the CPEs had undergone. This could explain why protease and cellulase activities were highest for most of the 5<sup>th</sup> CPE samples, and why amylase activity was observed only in the 5<sup>th</sup> CPE for the 90% corn meal feed blend. No lipase activity was detected among the crude protein extracts.



**Fig. 4-3 Cellulase Activity of Concentrated Crude Protein Extracts Preserved with Glycerol**

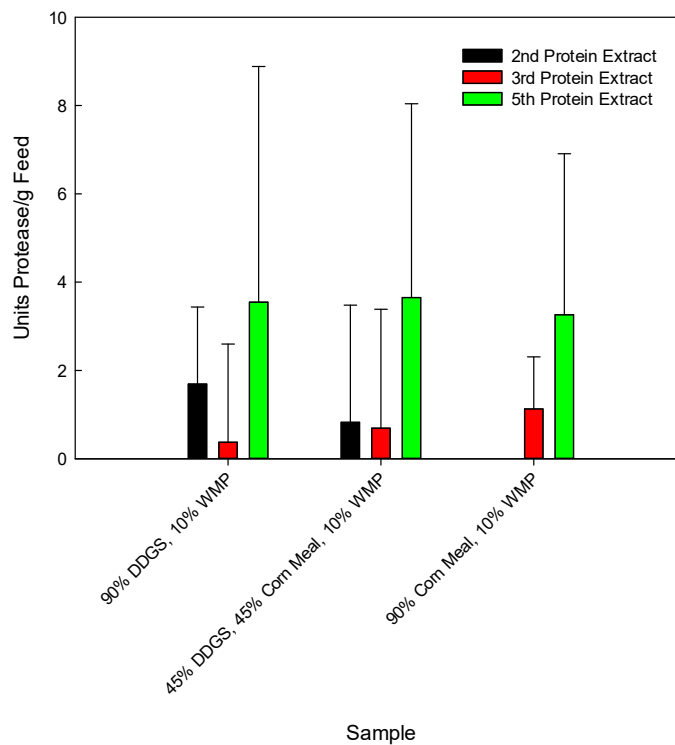
Feed blends were made of DDGS, cornmeal and whole milk powder. After BSFL digestion, the spent feeds were extracted with 50 mM Tris-HCl buffer with 100mM NaCl, pH 7.5. The extracts were numbered in the order they were made, so higher numbers mean that the extracts were made at a later date during larval digestion of feeds. N = 3; mean and std. dev.;

The overall findings of the screening of spent larval feeds for general enzyme activity indicates that there may be a low levels of amylase and cellulase present, but that their activity in spent feed is not consistent across DDGS concentrations or timeframes (as indicated by the order of CPE manufacture) of larval digestion.

Since the observed enzyme activities were produced by the BSFL’s digestive tract, which is colonized by bacteria, the enzyme activities in digested feeds may be the result of bacterial inoculation of the feed bed.

#### 4.3.2 Isolation of Potential Cellulolytic Symbionts from Spent Larval Feeds

The digested feed beds of the BSFL were analyzed for culturable bacteria which could contribute to feed degradation. **Table 4-1** shows the results of the sequencing of microbial isolates from spent larval feed. The four distinct organisms which were isolated were *Lactobacillus plantarum*, *Sphingomonas melonis*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae*. Each of the extracted 16S rDNA sequences showed high sequence identity to characterized sequences among sequence libraries using the BLAST alignment tool, except for culture #12.



**Fig. 4-4 Protease Activity of Concentrated Crude Protein Extracts of Spent Larval Feed**

Feed blends were made of DDGS, cornmeal and whole milk powder. After BSFL digestion, the spent feeds were extracted with 50 mM Tris-HCl buffer with 100mM NaCl, pH 7.5. The extracts were numbered in the order they were made, so higher numbers mean that the extracts were made at a later date during larval digestion of feeds. N = 3; mean and std. dev.; Means not significantly different at  $\alpha = 0.05$ ;

Since these species were identified in digested substrate, they may play an important role in the digestive abilities of the BSFL. It is acknowledged that many microorganisms in the BSFL gut may be unculturable by traditional methods, or may be fastidious, severely limiting their

isolation and identification by the above methodology. For industrial applications however, only culturable bacteria would be of practical use. *Lactobacillus plantarum* was the most frequently isolated organism, accounting for 50% of all isolates (Table 4-1).

**Table 4-1 Bacterial Isolates from Spent Larval Feed Identified by 16S rDNA Sequencing**

Culture #	Oxygen Growth Conditions	Genus	Species	Identity (%)
1	Aerobic	<i>Lactobacillus</i>	<i>plantarum</i>	99
2	Anaerobic	<i>Lactobacillus</i>	<i>plantarum</i>	99
3	Anaerobic	<i>Lactobacillus</i>	<i>plantarum</i>	99
4	Aerobic	<i>Sphingomonas</i>	<i>melonis/aquatilis</i>	98/98
5	Anaerobic	<i>Lactobacillus</i>	<i>plantarum</i>	99
6	Anaerobic	<i>Lactobacillus</i>	<i>plantarum</i>	99
7	Aerobic	<i>Pseudomonas</i>	<i>sp.</i>	99
8	Anaerobic	<i>Pseudomonas</i>	<i>fluorescens</i>	100
9	Anaerobic	<i>Klebsiella</i>	<i>pneumoniae</i>	99
10	Aerobic	<i>Sphingomonas</i>	<i>melonis/aquatilis</i>	97/97
11	Anaerobic	<i>Lactobacillus</i>	<i>plantarum</i>	99
12	Anaerobic	<i>Klebsiella/Enterobacter</i>	-	-

*Lactobacillus plantarum* is a gram-positive, aerotolerant bacterium. In the present study, *L. plantarum* was isolated under anaerobic conditions five times, and aerobic conditions once, confirming its anaerobic predisposition, as well as its aerotolerant ability. During anaerobic fermentation, *L. plantarum* produces lactic and acetic acids via the Embden-Myerhof Pathway, this reduces microbial competition in the substrate. It also has the ability to liquefy gelatin, and is often used as a fermentation organism in for foods, pharmaceuticals, and most pertinent to this study for silage. *Sphingomonas* spp. are gram-negative, rod-shaped, chemoheterotrophic, strictly aerobic bacteria. They were isolated here exclusively under aerobic growth conditions. *Sphingomonas* is notable for its ability to degrade hydrophobic, polycyclic, aromatic, hydrocarbons such as those used in polymeric plastics. Their increased hydrophobicity is due to a modified form of the lipopolysaccharide (LPS) layer typically found in gram-negative bacterium which uses a shorter carbohydrate structure <sup>212</sup>. *Pseudomonas* spp. are gram-negative, rod-shaped bacterium. While most *Pseudomonas* are obligate aerobes, certain strains of *P. fluorescens* are able to use nitrate instead of oxygen as a final electron acceptor during cellular respiration. It is not therefore surprising that the species was identified from both an aerobic and an anaerobic culture. Finally, *Klebsiella pneumoniae* is a gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It is normally

found on the skin, mouth, and intestines of humans, but is a pathogenic organism. It was isolated twice from anaerobic conditions, which is unsurprising given its identity as a facultative anaerobe. *Klebsiella*, *Psuedomonas*, and *Sphingomonas* all belong to the Proteobacteria phylum, while *Lactobacillus* belongs to the Firmicutes phyla. These bacteria were all isolated from larval digested substrates containing primarily corn meal.

In a study by Jeon and others, pyrosequencing of 16S rDNA was used to identify the gut microflora in BSFL fed on either a food-waste, calf-forage, or cooked rice diet<sup>164</sup>. The cooked rice diet most closely represents that of the diet used in the present study. The microbiome of the BSFL was shown to shift with the BSF's diet, with the food-waste diet showing the greatest microbial diversity, likely due to its higher nutrient diversity. The bacterial phyla identified from the gut of the cooked rice fed BSFL was Proteobacteria (54.01%), Firmicutes (42.47%), and unclassified (3.52%). *Psuedomonas*, *Klebsiella*, and *Sphingomonas* are Proteobacteria, while *Lactobacillus* are Firmicutes. While the present study used a less robust surveying method than the study by Jeon and others, the organisms isolated here from the gut of a separate population of BSFL matched the phylum/divisions present in their study. This suggests that they are autochthonous organisms, and may play a significant role in the digestive abilities of the BSFL. Given that the bacterial diversity was higher in more nutrient diverse diets, the corn-meal diet, while sufficient for larval growth, may be retarding their growth by limiting the microbial diversity in the insect's gut. Additional research by Zheng and others further elucidated the microbial community of the BSFL by measuring its shift over different life-cycle stages of the insect. Pyrosequencing of 16S rDNA was utilized on BSFL reared on Gainesville diet (20% corn meal, 30% alfalfa meal, and 50% wheat bran). They identified the presence of *Lactobacillus*, and *Psuedomonas* genera from the BSFL, as well as some members of the Shingomonadales order to which *Sphingomonas* belongs. Neither *Sphingomonas* nor *Klebsiella* were identified, but other Enterobacteriaceae including *Citrobacter* and *Eschericia* were present, suggesting that *Klebsiella* is not an unusual isolate for spent BSFL feed in the current study. The research by Zheng and others showed that the relative sequence abundancies across life stages, at the phylum level, changed very little between larvae, pre-pupae, and pupae. The adult and egg life-cycle stages showed the presence of additional bacterial phyla, but these life-cycle stages were not used for bacterial isolation in the present study. In the larval stage, Firmicutes and Proteobacteria were responsible for ~20% and ~15% of the relative sequence abundance respectively<sup>165</sup>. After the



conclusion of this experiment, it was learned that lactobacillus MRS media contains sodium acetate, which suppresses the growth of many competing bacterial and fungal species. This may also explain why the number of unique isolates was so low in the present study. The work presented here gives supporting evidence to the theories that the gut microflora of the BSFL is made up of both allochthonous (transient), and autochthonous bacterial species, and that the BSFL gut microbiome shifts depending on the diet provided.

**Table 4-2** summarizes the enzyme activities of bacteria isolated from spent larval feed. The cellulase activity was the highest for isolate #7, followed by isolate #4. Due to the low activity of extracellular enzymes in these isolates there was some imprecision in the amylase and cellulase results. All positive means with a coefficient of variation greater than 100% were assumed to be zero. Amylase activity was only observed in isolate #9. Protease activity was highest in isolate #3, followed by #5, #2, #7, #4, and #1 from highest to lowest. Lipase activity was very limited, being seen in only 2 of the 12 isolates, #11 and #12. Isolate #12 showed much greater lipase activity than isolate #11.

**Table 4-2 Enzyme Activities of Bacterial Isolates of Spent Larval Feed**

Culture #	Species Identity	Cellulase	Amylase	Protease	Lipase
		(Units/CFU) Mean ± Std. Dev.	(Units/CFU) Mean ± Std. Dev.	(Units/CFU) Mean ± Std. Dev.	(Units/C FU)
1	<i>Lactobacillus plantarum</i>	0	0	0	0
2	<i>Lactobacillus plantarum</i>	0	0	3.48E-04 ± 1.83E-04	0
3	<i>Lactobacillus plantarum</i>	0	0	7.50E-04 ± 2.14E-04	0
4	<i>Sphingomonas melonis/aquatilis</i>	4.49E-07 ± 7.49E-08	0	2.23E-11 ± 1.45E-11	0
5	<i>Lactobacillus plantarum</i>	0	0	4.07E-04 ± 6.13E-04	0
6	<i>Lactobacillus plantarum</i>	0	0	0	0
7	<i>Pseudomonas sp.</i>	8.55E-06 ± 7.17E-06	0	4.19E-10 ± 8.49E-11	0
8	<i>Pseudomonas fluorescens</i>	0	0	0	0
9	<i>Klebsiella pneumoniae</i>	0	7.06E-10 ± 2.35E-10	0	0
10	<i>Sphingomonas melonis/aquatilis</i>	0	0	0	0
11	<i>Lactobacillus plantarum</i>	0	0	0	3.57E-11
12	<i>Klebsiella/Enterobacter</i>	0	0	0	0.018

N = 3;

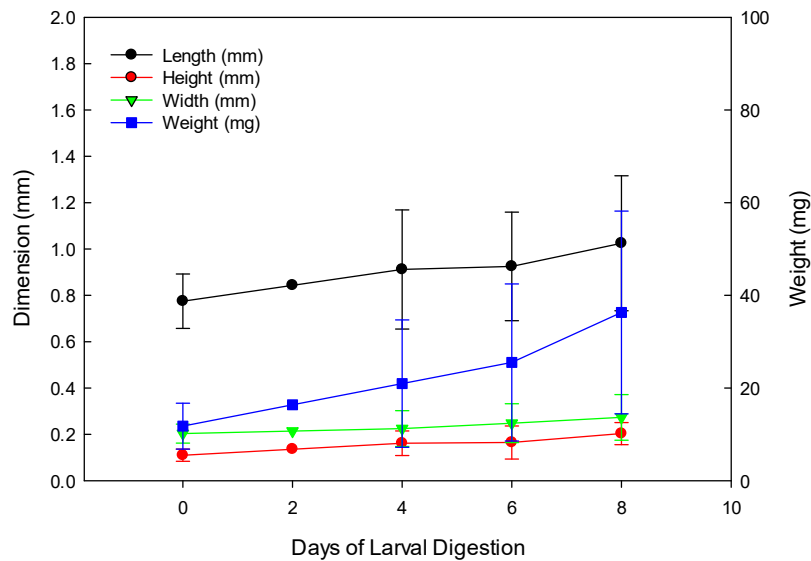
Cellulase activity was limited to only a couple of bacterial isolates, one of two *Sphingomonas* isolates, and one of two *Pseudomonas* isolates. Lipase activity was also limited, being detected in only one of six *Lactobacillus* and one of two *Klebsiella* isolates. Amylase activity was also rare, being present only in one of two isolates for *Klebsiella*. Protease activity

was the most widely distributed among isolates four of six *Lactobacillus*, one of two *Sphingomonas*, and one of two *Klebsiella* isolates displaying activity.

*Lactobacillus plantarum* was also the most abundant isolate identified from the feed beds and has been used in previous SSF operations. The *Lactobacillus plantarum* isolates had the greatest protease activity. Therefore among the isolates shown here, this organism showed the greatest potential for DDGS pretreatment before the addition of larvae. This subject was evaluated in Study 2 which included the use of solid state fermentation on DDGS substrate. In addition to proteases, *L. plantarum* is known to produce some cellobioses such as  $\beta$ -glucosidase, but is not known to produce cellulase naturally.

### 4.3.3 Lignocellulose Feed Content Effect on Enzyme Activity

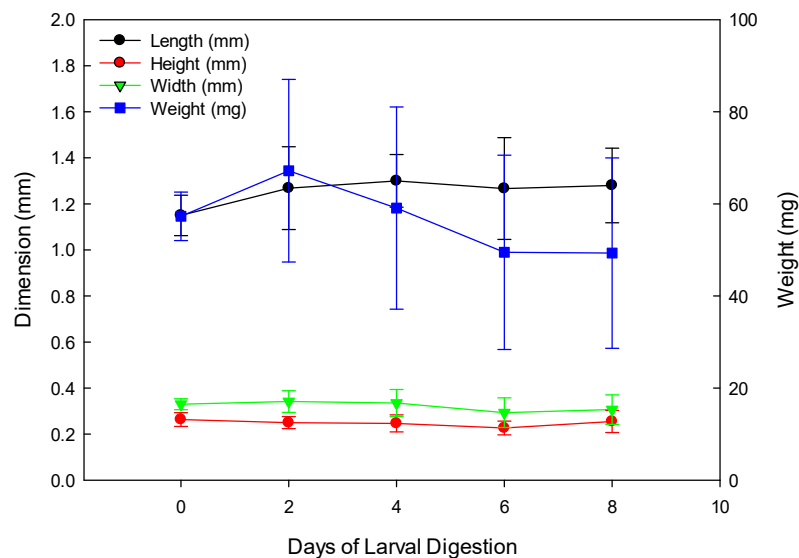
In order to assess the effect of cellulose on the digestive abilities of BSFL synthetic feed blends were made of various cellulose concentrations which were then subjected to larval digestion. The concentration of cellulose in the synthetic feed blend of microcrystalline cellulose and corn meal was not strongly correlated to the growth of larvae. **Table 4-3** summarizes the larval parameter changes during 8 days of larval culture shown in **Figs. 4-5** to **4-9**. From **Table**



**Fig. 4-5 Larval Parameters of 0% Cellulose Feed Culture**

Six larvae were randomly chosen and measured to check their growth. N = 6; mean and std. dev.

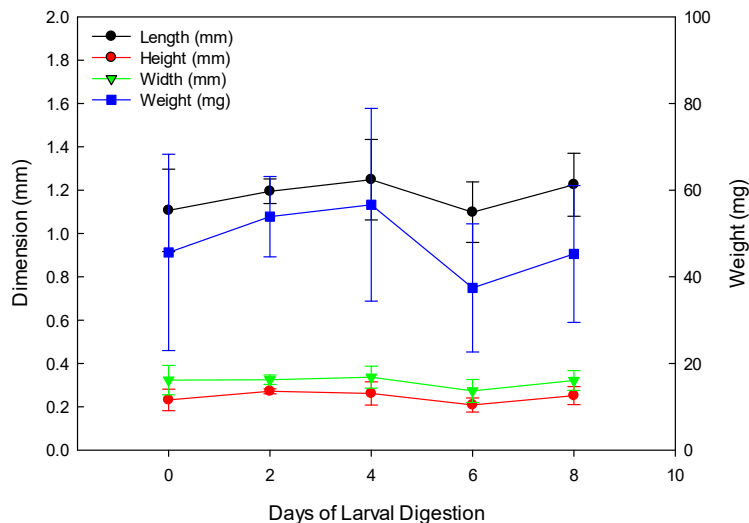
4-3 all larval parameters increased during the 8 day larval digestion of the 0% cellulose feed substrate. Although the numerical value of larval height and width did not increase much, the weight change as a percentage of the starting dimensions was very high at 208.2% increase. This indicates that larvae can grow efficiently on a blend of 90% corn meal/10%WMP. For the 25% cellulose sample, the larval dimensions and weight all decreased over the 8 day digestion period, except for length which saw a slight increase from days 0 to 8. For the 50% cellulose sample, the larval dimensions and weight fluctuated greatly during the 8 day larval digestion period. Length and height saw an overall increase, while width and weight saw a decrease. And lastly, larval parameters increased during digestion of both the 75% and 100% cellulose samples.



**Fig. 4-6 Larval Parameters of 25% Cellulose Feed Culture**

Six larvae were randomly chosen and measured to check their growth. N = 6; mean and std. dev.

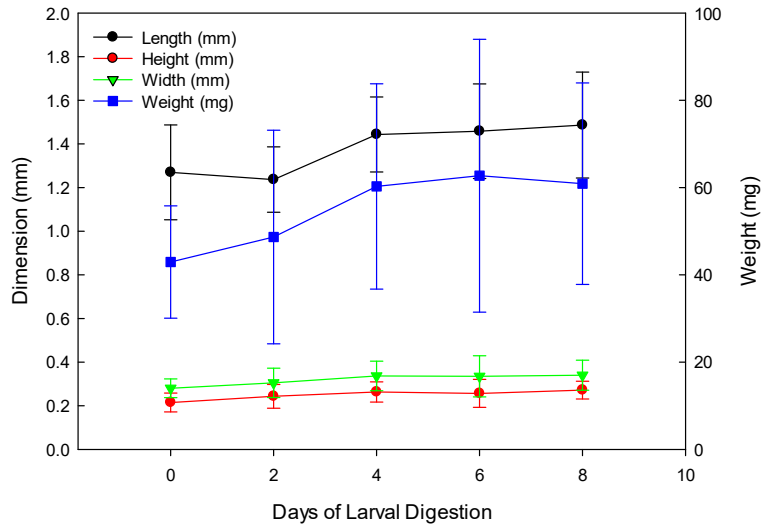
The larger weight increase for the 0% cellulose sample compared to other cellulose feed blends can be explained by the age of the population of larvae used for that sample. Medium sized larvae were obtained and utilized in the 0% cellulose sample digestion, and then subsequently for later samples containing higher cellulose concentrations. This means that the larvae used for higher cellulose content feeds were older and closer to their maximum potential size. They had already realized a lot of their juvenile size and weight gain. On **Fig. 4-5** it is seen that the larvae had an average weight of 10 mg at the start of the larval digestion period, or day 0 for the 0% cellulose sample. **Fig. 4-6** shows that the larvae used in the 25% cellulose sample had



**Fig. 4-7 Larval Parameters of 50% Cellulose Feed Culture**

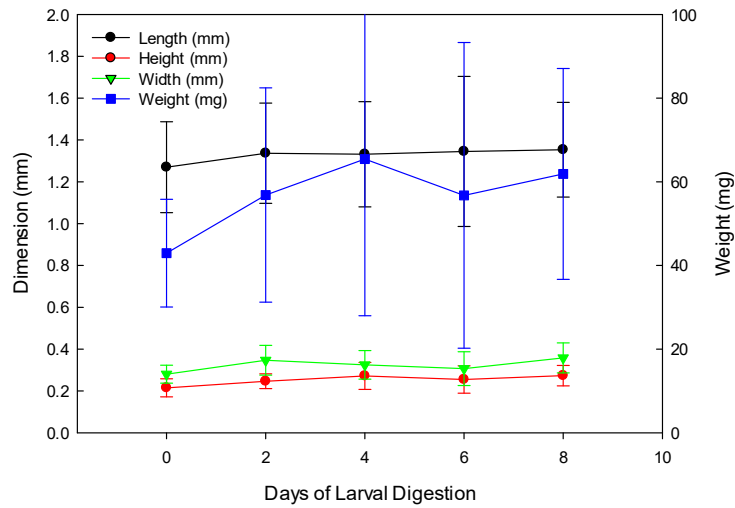
Six larvae were randomly chosen and measured to check their growth. N = 6; mean and std. dev.

an average starting weight of nearly 60mg, and then saw a loss in weight. And the remaining three samples had average starting larval weights of nearly 40mg as seen on **Figs. 4-7 to 4-9**. These average weight values have very large standard deviations however, given that only 6 larvae were measured out of a much larger sample size. Therefore these weight change measurements may not accurately reflect the change in the total larval population. It was expected that samples containing higher concentrations of cellulose would limit the growth of larvae given its lower nutrient availability, but when considering the 25-100% cellulose samples the opposite trend was seen. From **Table 4-3** the weight change during the larval digestion period for the 25% and 50% cellulose feed blends was negative, while for the 75% and 100% cellulose feed blends it was positive. This is likely a reflection of the starting age of the larvae for each sample. BSFL are capable of growth in high cellulose feed blends, which indicates that they do possess some ability to degrade and digest cellulose. The 0% cellulose sample provided the best environment for larval biomass growth through a combination of more nutrient dense and bioavailable substrate, and by starting with very young larvae.



**Fig. 4-8 Larval Parameters of 75% Cellulose Feed Culture**

Six larvae were randomly chosen and measured to check their growth. N = 6; mean and std. dev.



**Fig. 4-9 Larval Parameters of 100% Cellulose Feed Culture**

Six larvae were randomly chosen and measured to check their growth. N = 6; mean and std. dev.

**Table 4-3 % Change in Larval Parameters during 8 Day Larval Digestion Cellulose Synthetic Feed Blends**

Parameter	% Change 0% Cellulose	% Change 25% Cellulose	% Change 50% Cellulose	% Change 75% Cellulose	% Change 100% Cellulose
Length (mm)	32.3	11.3	10.7	17.1	6.6
Height (mm)	84.8	-3.2	8.6	26.4	27.1
Width (mm)	34.4	-7.1	-0.5	21.4	28.0
Weight (mg)	208.2	-13.9	-0.8	41.8	44.1

From **Table 4-4**, the protein concentration of the CPEs of the 0% cellulose feed substrate increased during the larval digestion period. Similarly, both the 25% and 50% cellulose sample's CPEs showed an increase in protein concentration over the 8 day larval digestion period. Protein concentrations of the CPEs from the 75% cellulose sample decreased over the larval digestion period, which is contrary to the trend observed for the 0%, 25%, and 50% cellulose samples. The protein concentration of CPEs prepared from the 100% cellulose sample showed no trend over the larval digestion period. The protein concentration of the CPE decreased as the cellulose content of the larval feed increased.

**Table 4-4 Protein Concentration of Cellulose Synthetic Feed Blends**

Days Larval Digestion	0% Cellulose Protein Conc. (mg/ml)	25% Cellulose Protein Conc. (mg/ml)
0	-	-
2	0.174 ± 0.009	0.085 ± 0.014
4	0.164 ± 0.007	0.087 ± 0.015
6	0.184 ± 0.027	0.084 ± 0.012
8	0.210 ± 0.023	0.208 ± 0.006

N = 3; means and std. dev.;

**Table 4-5** summarizes the effect of feed cellulose concentration on enzyme activities in digested larval substrates over the larval digestion period. No cellulase was observed from the spent feed of 0% cellulose. In general, enzyme activity decreased over the larval digestion period for this sample, but the highest activity was observed in days 6, 4 and 4 for amylase, lipase, and protease respectively. Cellulase activity was observed after 2 days of larval digestion of the 25% cellulose substrate, but not on any other day. No cellulase activity was observed for the 50%, 75%, and 100% cellulose samples. It does not therefore appear that cellulase is present at sufficient levels in spent feed to efficiently degrade the cellulose therein. Therefore the use of

additional enzymes could be of practical use when raising larvae on fibrous feeds. This hypothesis was addressed here in Study 2.

Amylase activity, shown on **Table 4-5**, showed high activity in the 0% cellulose sample on days 2 and 6 of larval digestion, but no overall trend was established given that the activity level decreased again by the 8<sup>th</sup> digestion day. The observed amylase activity decreased as the cellulose content of the sample increased, and was only observed again on day 12 of the 25% cellulose sample. Additionally, the precision of the measurements became much less as the activity level decreased. This suggests that this method has a higher limit of detection. At very low activity readings, the absorbance values from the sample are hardly distinguishable from the blank values, and precise measurements become difficult. No amylase activity was observed at cellulose concentrations of 50% and above.

From **Table 4-5**, lipase activity was observed in all samples except for 100% cellulose. Day 4 of the 0% cellulose sample showed the overall highest lipase activity. Lipase activity was the greatest on day 6 for the 25% cellulose substrate, but no trend was evident for lipase in that sample. The 50% cellulose substrate showed a steady increase in lipase activity during larval digestion, with the highest activity observed on day 8. The lipase activity levels were much lower than those observed for the 25% and 50% samples, but were higher than those observed from the 0% cellulose sample. Lipase activity was observed on all days from the 75% cellulose sample, but not at all in the 100% cellulose sample.

From **Table 4-5**, protease measurement delivered precise measurements for samples, and presented strongly in the 0% cellulose sample. The greatest overall protease activity was observed at day 4 for the 0% cellulose sample. Protease activity was observed in on days 6 and 8 for the 25% cellulose sample, and days 4, 6, and 8 for the 50% cellulose sample. No protease activity was observed in the 75% cellulose sample. Similar to the other assays, as the protease activity level approached zero such as occurred in the higher cellulose concentration samples, the precision was reduced. No enzyme activity was observed on any day of larval digestion of the 100% cellulose sample.

The overall conclusion of the study of enzymes isolated from spent larval feeds is that while there may be some small activity yet present after the substrate has passed from the larval gut, it is a negligible amount. Study 1 proves the first hypothesis of this research, that the concentration of enzymes in spent larval feed is dependent upon the composition of the starting

feed. Enzyme activity in spent larval feed is the most present after several days of digestion of the 0% cellulose sample. This indicates that the larvae are more digestively active when provided with a more nutritious feed source, and that 100% cellulose is a poor substrate for larval rearing.

**Table 4-5 Enzyme Activity of Cellulose Synthetic Feed Blends**

Sample	Days Larval Digestion	Cellulase (U/g Feed)	Amylase (U/g Feed)	Lipase (U/ g Feed)	Protease (U/g Feed)
0% Cellulose	0	-	-	-	-
0% Cellulose	2	0	3.14 ± 0.46	0.19 ± 0.10	4.40 ± 0.19
0% Cellulose	4	0	0	52.29 ± 11.56	22.62 ± 8.37
0% Cellulose	6	0	12.18 ± 0.95	0	6.25 ± 5.89
0% Cellulose	8	0	0.11 ± 0.17	0	0.27 ± 0.02
25% Cellulose	0	-	-	-	-
25% Cellulose	2	190.47 ± 107.63	0	1.70	0
25% Cellulose	4	0	0	1.70	0
25% Cellulose	6	0	0	4.08	0.70 ± 0.15
25% Cellulose	8	0	0.21 ± 0.015	0.70	0.37 ± .08
50% Cellulose	0	-	-	-	-
50% Cellulose	2	0	0	1.00	0
50% Cellulose	4	0	0	1.73	0.33 ± 0.27
50% Cellulose	6	0	0	1.71	0.50 ± 0.12
50% Cellulose	8	0	0	7.82	0.39 ± .015
75% Cellulose	0	-	-	-	-
75% Cellulose	2	0	0	0.41 ± 0.33	0
75% Cellulose	4	0	0	0.24 ± 0.11	0
75% Cellulose	6	0	0	0.51 ± 0.21	0
75% Cellulose	8	0	0	0.15 ± 0.09	0
100% Cellulose	0	-	-	-	-
100% Cellulose	2	0	0	0	0
100% Cellulose	4	0	0	0	0
100% Cellulose	6	0	0	0	0
100% Cellulose	8	0	0	0	0

N = 3; mean and std. dev.;

#### 4.4 Connection to Next Study

Anecdotal evidence suggests that BSFL lacked a strong mechanism for the degradation of cellulose itself. Past research on BSFL shows the larvae have high enzyme activities in their digestive tract, but the present study showed the enzymes did not survive in their secretion well. Therefore, enzyme contribution from BSFL would have limited effects on the processing of the



cellulosic feedstuff. It may therefore be beneficial to pre-treat cellulosic materials prior to digestion by the BSFL in order to maximize both dry matter reduction of substrates and bioconversion of substrates into larval biomass. Pretreatment of DDGS through SSF and size reduction of the substrate are used in Study 2 and the effect on bioprocessing and bioconversion of DDGS is evaluated.

## 5 STUDY 2: LIGNOCELLULOSE FEED CONTENT EFFECT ON SUGAR PRODUCTION

### 5.1 Abstract

It is investigated whether the use of solid state fermentation (SSF) could improve the overall nutrient profile for BSFL culture and increase the recovery of fermentable sugars in combination with BSFL culture. SSF using several fungal strains known for their cellulolytic abilities and *Lactobacillus plantarum* isolated from the BSFL were used as a pre-treatment of DDGS, prior to larval digestion. SSF was shown to open the DDGS structure; reduce the cellulose content by around 5 percent; decrease the total carbohydrate content by up to 13 percent; and increase the protein concentration by up to 8 percent. SSF using *Aspergillus niger* had the greatest effect on DDGS, reducing the carbohydrate content by 13 percent, and increasing the protein content by 8 percent. Following this analysis, we investigated whether fermented DDGS was suitable for larval feeding, and the effect that BSFL digestion had on the fermentable sugar recovery from DDGS. Digestion of fermented DDGS by BSFL reduced the dry matter of the substrate and incorporated it into a high protein (37% dwb), high fat (23% dwb) larval biomass. Considering DDGS contains very low fat (2%), the significant fat recovery/yields can be achieved through SSF-BSFL treatment. When untreated DDGS was given to BSFL, the larvae can be incorporated 43.7 % of dry mass of DDGS; whereas SSF DDGS can be incorporated up to 80.4% after the digestion by a combination of SSF and BSFL culture. The relative carbohydrate contents of the substrates were increased during larval digestion which, along with opening of the DDGS constituent matrices during treatments, allowed for increased glucose liberation from the substrates in spent feeds. Optimization of hydrolysis conditions, including higher temperatures, pressure, and substrate loading concentration, further provided large increases in total glucose yields compared to unfermented DDGS (8 % of dry weight of DDGS carbohydrates), particularly for *Lactobacillus plantarum* SSF (16% of dry weight of DDGS carbohydrates) (Fig. 5-34).

## 5.2 Experimental approach

### 5.2.1 Materials

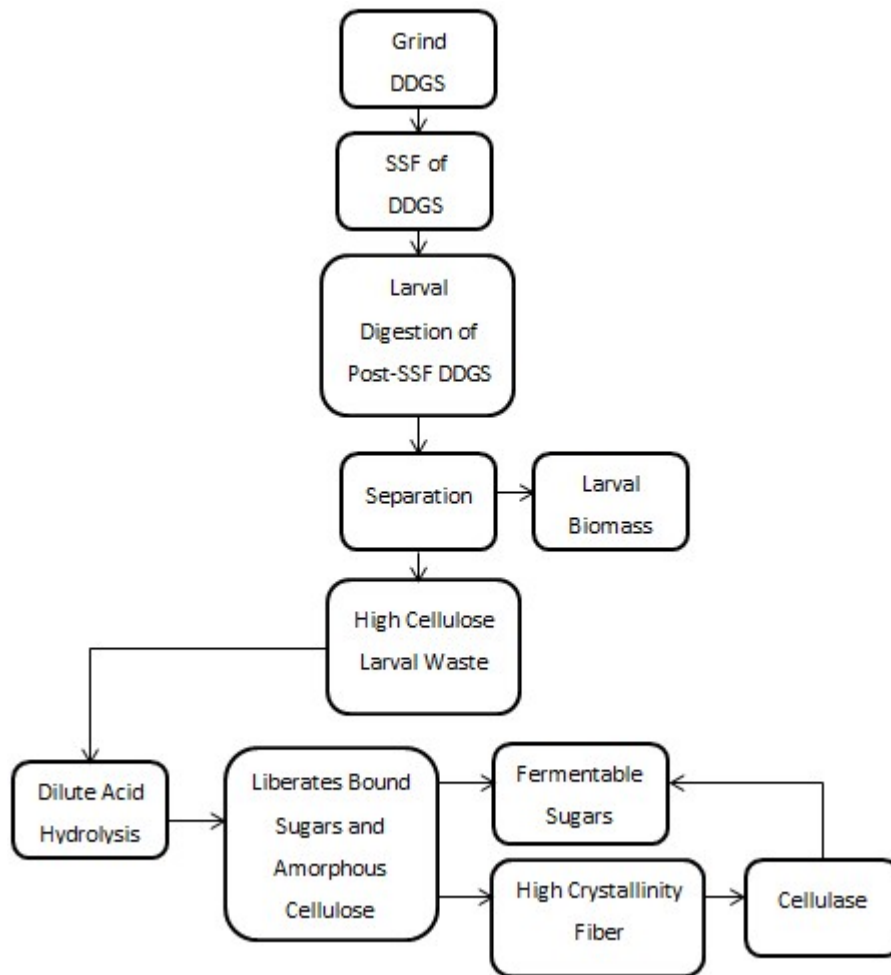
All fungal strains were obtained from the ARS Culture Collection (Bacterial Foodborne Pathogens and Mycology Research Unit; National Center for Agricultural Utilization Research; Peoria, IL USA). *Lactobacillus plantarum* used for SSF was obtained from stock cultures of isolate #5 from Study 1 presented here. Glucose concentration was analyzed using the Glucose (HK) Assay Kit from SigmaAldrich (St. Louis, MO, USA). All chemicals used in this study were commercially available ACS grade, and were purchased from VWR International (Edmonton, AB) and Fisher Scientific (Ottawa, ON).

### 5.2.2 Acid Pretreatment and Enzymatic Hydrolysis of Digested Lignocellulosic Feed

The sugar liberation from lignocellulosic materials was examined as shown in (**Fig. 5-1**). The DDGS was fed to BSFL and the samples were withdrawn every three days up to and including day 12 of larval digestion. The samples were examined for the production of fermentable liberated sugars, as approximated by glucose concentration. The determination of liberated sugars was conducted at two stages<sup>190</sup>: acid and enzymatic hydrolysis.

The acid hydrolysis conditions were as follows: the sample was suspended at 60 mg/mL in 2% HCl, and the suspension was incubated at 90 °C for 3 hours. The suspension was centrifuged (12,000 xg, 20 min, room temperature) to separate the suspension into liquid and solid parts. The amount of free glucose was measured via hexokinase/glucose-6-phosphate dehydrogenase system measured spectrophotometrically<sup>202</sup>. The sample was suspended in a buffered solution (pH 7.6), and was then incubated with hexokinase and glucose-6-phosphate in the presence of NADH. Free glucose was phosphorylated by hexokinase, which was powered by the dephosphorylation of ATP, and the resulting glucose-6-phosphate was reduced to 6-phosphoglucanate oxidizing NAD<sup>+</sup> to NADH. The NADH generated by this process is linear in concentration to amount of glucose and was measured through spectrophotometric absorption at 340 nm. The results primarily indicate the amount of amorphous cellulose on the cellulose particles following BSFL digestion.

The solid matter after acid treatment was washed with water several times to a neutral pH. The solid parts represent the remaining crystalline cellulose which has been micronized from the BSFL mastication. The solid part was loaded into flasks and suspended 1% (wt. substrate/v.) in sodium acetate buffer (pH 4.8). Cellulase was then added at 20 mg enzyme/g (wet-weight basis) of solid. Tetracycline was added for bacterial control at 40 µg/ml. The hydrolysis was carried out



**Fig. 5-1 Process Flow Chart for the Bioconversion of DDGS**

Solid state fermentation (SSF) and larval digestion by the Black Soldier Fly larvae both produce fermentable sugars through the hydrolysis of starch, and the hydrolysis of amorphous cellulose from cellulose particles. This increases the glucose concentration of High Cellulose Larval Waste here. Dilute acid hydrolysis produces glucose via the hydrolysis of starch, and of amorphous cellulose. Free glucose is subtracted from the glucose yields from dilute acid hydrolysis, so acid hydrolysis is primarily a measure of amorphous cellulose, and/or starch in the sample. Following dilute acid hydrolysis, enzymatic hydrolysis (Cellulase step here) produces glucose by enzymatically cleaving it from crystalline cellulose. As free glucose, starch, and amorphous cellulose were removed from the substrate in the preceding steps, the enzymatic hydrolysis step is a measure of the sugars which can be recovered from the crystalline fractions of cellulose under the current system. The digested glucose, and other biomass components such as proteins are partially recovered in the form of high quality larval biomass.

in an incubator shaking at 50°C and 150 rpm for forty-eight hours. This enzymatic step liberates the glucose from the crystalline fraction of the cellulose particle. The sugar amounts of the two stages in addition to free pre-hydrolysis sugars are considered as the total amounts of sugar that can be obtained through the BSFL culture.

### 5.2.3 Solid State Fermentation and Larval Digestion of Lignocellulosic Biomass

A variety of companion microbes either isolated from the BSFL in Study 1 and/or obtained from culture deposits will be examined in 'co-fermentation' with BSFL. The eight fungal strains analyzed included: two sup-species of *Aspergillus niger*, two sub-species *Aspergillus fumigatus*, *Trichoderma reesei*, *Phanerochaete chrysosporium*, *Trichoderma koningii*, and *Trichoderma viride*.

The organisms suitability for growth and substrate degradation using 100% DDGS was first analyzed. Stock culture collections of each organism were made by growing each strain on Sabouraud agar plates until sporulation was seen. The spores were collected using mechanical disturbance, and mixing with a small amount of sterile water. The spore suspension was mixed 1:1 with sterile glycerol, and the mixture was placed in -80°C storage.

In order to evaluate the effect of particle size on SSF, DDGS was ground using a knife mill and fractionated by size. Particle size fractionation was performed by sieving. The ground DDGS was separated after sieving into fractions greater than 0.0139 inches in diameter called coarse fractions, and those fractions which were less, called fine fractions. The majority of the ground DDGS was not sieved or separated and will be henceforth referred to as homogeneous DDGS to differentiate it from the fine and coarse fractions. One milliliter of spore suspension without glycerol was used to inoculate 20 g moist DDGS of fine, homogeneous, or coarse particle size. The samples were held in closed 50 ml containers at 30 °C. At time intervals, the DDGS was observed via visual inspection for the following: DDGS coverage, hyphal growth, and sporulation presence. After 25 days, the moisture content, cellulose content, crystallinity index of the extracted cellulose, and acidic and enzymatic sugar liberation were measured as previously outlined. Based on the results of the preliminary SSF experiment *Trichoderma koningii*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Phanerochaete chrysosporium* fungal strains, and *Lactobacillus plantarum* isolated from the spent larval media in Study 1 were chosen for further analysis in combination with larval growth.

Three day seed cultures of selected fungal strains were prepared by inoculation of 50mL of potato dextrose media in 250 mL Erlenmeyer flasks, followed by 72 hr incubation at 30 °C and shaking at 150 rpm. Fifty grams of sterile homogeneous blend ground DDGS was adjusted to 75% moisture to which was added 1.2% urea (dry weight of urea/dry weight of feed) to facilitate microbial catabolism. Sixteen milliliters of seed culture was added to the DDGS after the addition of water and urea. The samples were fermented at 30 °C, and were mixed once per day using aseptic technique.

The samples were prepared inside of Tupperware containers with small holes placed all around the lid to allow moisture and gas transfers during SSF. These Tupperware containers were all placed into a larger container, ensuring to not obstruct ventilation holes. Small containers of water were also placed inside of the larger plastic container to maintain high humidity. The relative humidity was 75% ( $\pm 7\%$  according to manufacturer's specifications), which was measured using Accu-Temp brand Humidity Meter. At the conclusion of SSF, all of the samples were frozen until sufficient larvae became available for larval digestion. Samples were thawed prior to larval addition by heating to equilibration with the room temperature by placing them on the lab bench.

One hundred grams (wet weight basis) of larvae were placed into the thawed post-SSF substrate. At days 0, 3, 6, 9, and 12 of larval digestion the weight change of the system was measured, and a portion of the sample and larvae were both removed to test the following: moisture content of the substrate; moisture content of the larvae; larval body dimensions and weight; acidic and subsequent enzymatic hydrolysis of the extracted substrate for sugar liberation; cellulose content; and crystallinity index (CI) of the extracted cellulose. The moisture content was measured by placing ~1 g of substrate into pre-weighed microfuge tubes, and drying overnight at 80 °C to a constant weight. The loss in weight of the sample is equal to the moisture content of the sample.

Weights of the empty container, container with substrate after SSF, and container with substrate and larvae were all taken. The same scale was used throughout the process for these measurements. A sample of both SSF and larvae were removed every three days up to and including the 12<sup>th</sup> day of larval digestion. At the 12<sup>th</sup> day, all larvae and substrate were removed from the container. Care was used to take a representative sample of larvae and substrate so as not to change the larvae-to-substrate ratio present at any given testing day. The container was

weighed on each testing day before the removal of any constituents. The weight of the removed substrate and larvae were measured. The larvae were removed from the sample using a weighing spatula, taking care not to damage the larvae. The larvae were rinsed off with distilled water, until the water ran clear, to remove any substrate on the outside of the larval bodies. These larvae were dried using a paper towel, and their weight was measured. This weight was subtracted from the weight of the removed sample to obtain the weight of the removed substrate. This data was used to calculate dry matter reduction of the substrate, as well as the utilization percent of the substrate, and incorporation of substrate into larval biomass.

Larval dimensions were measured on 6 randomly selected larvae from the sample of larvae removed from the system on that day. The length, width and height were all measured using hand calipers, taking special care to elongate the larvae prior to taking measurements. The sugar liberation assay was performed as indicated here in section 4.2.1.2 as a measure of the sugar liberation and total theoretical sugar yield.

The cellulose content was measured according to the methods of Brendel *et. al.*<sup>203</sup> with minor modifications. Approximately 1.5 g of substrate (wet-weight basis; wwb) was loaded into pre-weighed 25 mL centrifuge tubes with caps. Acetic acid (80%; v/v) was added at 2.0 mL/ g (wwb) substrate, and nitric acid (69%; v/v) was then added at 0.2 mL/ g (wwb) substrate. These acids served to de-lignify the substrate and hydrolyze non  $\alpha$ -cellulosic compounds. The method has been shown to produce little to no cellulose degradation, and no evidence of acid or aldehyde increases which would indicate oxidative degradation was observed<sup>203</sup>. The latter is a possible outcome of substrate exposure to aqueous nitrogenous oxidants<sup>204</sup>. The hydrolyzed substrate was then cooled, and mixed with 2.5 mL of 95% ethanol. The tubes were then sealed, mixed by vortexing, and centrifuged for 5 minutes at 5000 rpm (Sorval SS-34 rotor) to pelletize the cellulose.

After the initial ethanol wash, the following washes were used to remove the excess nitrogen content from the nitric acid, as well as the remaining hydrolyzed substrate constituents: 5 mL 95% ethanol, 5 mL deionised water, 5 mL 95% ethanol, and finally 5 mL acetone. Each wash was done in two 2.5 mL parts. The first volume was added, the substrate was mixed via vortexing, and then the second wash was used to wash the walls of the vessel free of substrate. After each wash, the sample was centrifuged, and the supernatant was removed via the vacuum apparatus. Following the acetone wash, the tubes containing the pelletized sample of  $\alpha$ -cellulose

and the cap were dried to a constant weight overnight at 80°C. The weight of the dried sample was equal to the cellulose content in the starting material.

The crystallinity index of the extracted cellulose was measured via the method of Hessler and Power<sup>205</sup> with a few modifications. Dried extracted cellulose (0.6 g) was treated with 2 mL of an iodine solution (5 g iodine, 40 g potassium iodide, and 50 mL deionized water) in a 125 mL Erlenmeyer flask. The sample was mixed for 1 minute, and then 100 mL saturated sodium sulfate was added to wash out excess iodine from the cellulose fibers. The sample was stored in a dark location for one hour, and then the remaining iodine in solution was measured by titrating a 1mL aliquot of the solution with a 0.02N sodium thiosulfate solution to which was added the following: 50 mL deionized water, 1 mL 1% (w/v) starch solution. The ratio of the amount of I<sub>2</sub> adsorbed per gram of cellulose to the maximum I<sub>2</sub> binding capacity of amorphous cellulose (methocel) gives a value of the amorphous fraction. The crystallinity was determined by subtracting this percent amorphous fraction from 100%. Methocel is a β-1,4 glucose polymer containing a methylated hydroxyl group which prevents crystalline structure formation, thereby becoming purely amorphous and capable of adsorbing the most I<sub>2</sub>. One gram of methocel absorbs 412 mg of iodine. Overall flow of the preparation of larval spent feed bed and its analysis is summarized in **Fig. 5-1**.

#### **5.2.4 Optimization of Sugar Liberation from SSF and Larval Digested DDGS**

Sterilized DDGS was used as the substrate to optimize parameters for dilute acid hydrolysis glucose yields. The parameters investigated were the following: substrate concentrations of 10-60 (g/100mL); acid concentrations of 2-10% (vol./vol.); acid type of HCl or H<sub>2</sub>SO<sub>4</sub>; reaction times of 0.5-48 hr.; and vessel types of either snap closure microfuge tubes (low/no pressure), or screw-cap microfuge tubes (high pressure). Glucose concentrations were determined using hexokinase/glucose-6-phosphate dehydrogenase system described in section 5.1.2. The optimum conditions for acid hydrolysis resulting from these experiments was 10% substrate concentration, in 2% HCl, 100°C for 3 hr. using screw-cap microfuge tubes. These parameters were used for the optimized glucose liberation acid hydrolysis experiments.

#### **5.2.5 Proximate Analysis of DDGS and Larvae from SSF and Larval Digestion**

Proximate analysis was conducted on BSFL, DDGS, post-SSF DDGS, and DDGS at 6 and 12 days of larval digestion by measuring the moisture content, crude fat, crude protein, ash



content, and total carbohydrates by difference. The moisture content was measured by placing ~1 g of substrate into pre-weighed microfuge tubes, and drying overnight at 80 °C to a constant weight. The loss in weight of the sample is equal to the moisture content of the sample. Crude fat was measured according to AACC method 30-20.01, Crude Fat in Grain and Stock Feeds<sup>206</sup>. The samples were dried to 0% moisture prior to analysis, and 0.5-1.0 g of sample was assayed in duplicate using Goldfish extraction for 6 hr with petroleum ether as the extraction solvent. The crude fat was equal to the weight of the lipids extracted after removal of the solvent. The ash content was measured according to AACC method 08-01.01, Ash-Basic Method<sup>207</sup>. Triplicates of 0.5-1.0 g of sample at 0% moisture was incinerated in a muffle furnace at 550 °C for 24 hrs. The ash content was equal to the weight of the non-combusted material after total combustion and cooling of the sample. Crude protein content was measured according to AACC method 46-30.01, Crude Protein-Combustion Method<sup>208</sup>. 0.2 g of sample was combusted in duplicate, and the resulting N was measured. A conversion factor of 6.25 was used to calculate total protein in the sample.

## **5.3 Results and Discussions**

### **5.3.1 Purpose of Acid Pretreatment and Enzymatic Hydrolysis of Digested Lignocellulosic Feed**

Study 1 found that BSFL are capable of digesting and degrading cellulose, but that higher cellulose content feeds have a retarding effect on BSFL digestive abilities. Additionally, it was shown that enzyme activities in digested larval feeds were not high and would therefore not likely result in passive feed degradation in the absence of direct larval digestion. In study 2, non-synthetic feed blends were used to simulate practical application of BSFL digestion of lignocellulosic feeds. DDGS starts as a dry, complex mixture of mainly proteins and carbohydrates in the form of lignocellulose. Most of this lignocellulose is hemicellulose, and is easily hydrolyzed into pentoses during dilute acid hydrolysis. A small amount is present as lignin, and twenty to thirty percent of carbohydrates are present as cellulose. SSF was evaluated as a means of enzymatic pretreatment of lignocellulosic feeds in Study 2.

In order to evaluate the recovery of fermentable sugars, three sequential processes of glucose liberation and determination were conducted: free glucose, acid hydrolysis, and enzymatic hydrolysis (**Fig. 5-1**). These represent glucose liberated through SSF and/or BSFL

digestion, glucose liberated from amorphous structured cellulose and/or starch, and glucose liberated from the crystalline structured cellulose, respectively.

#### 1: Free glucose

Free glucose may be generated in DDGS when BSFL chew up the cellulose particles. Some of the fibers are sheared off as linear  $\beta$ -1,4-glucose polymers. These are degraded further by enzymes in the BSFL digestive system, or by residual enzymes left over from the SSF period in the case of combined SSF and BSFL digestion. This glucose amount is considered as glucose derived directly from the SSF and BSFL actions.

#### 2. Amorphous glucose (acid hydrolysis liberation)

When BSFL chew up the cellulose particles, they mechanically micronize them, potentially disturbing crystalline regions of the cellulose. Some cellulose fibers remain attached to the greater particle as free hanging fibers, or non-hydrogen bound chains that link crystalline particles together. These are collectively called amorphous cellulose as they do not exhibit a crystalline structure. Amorphous cellulose is much more susceptible to hydrolysis than crystalline cellulose, and so dilute acid hydrolysis primarily measures the hydrolysis of amorphous cellulose into glucose.

#### 3. Crystalline glucose (enzymatic hydrolysis liberation)

Crystalline cellulose is less susceptible to acid hydrolysis. Thus the second process of glucose liberation (acid hydrolysis) largely leaves the crystalline cellulose. The remaining crystalline cellulose is susceptible to enzymatic hydrolysis. Therefore, enzymatic hydrolysis primarily measures the hydrolysis of crystalline cellulose into glucose.

SSF acts to increase free glucose by hydrolyzing cellulose into glucose through a blend of cellulases. This free glucose may be used for the metabolic activity of the SSF organism however, which can prevent increases in free glucose being observed. It also acts to disassociate lignocellulose components and overall DDGS matrices, primarily through protease activity. These allow for increased access of enzymes to the actual cellulose particles. BSFL digestion reduces particle size during chewing, and further increases enzyme access to cellulose through an increase in the particle surface area. In the Study 2 acidic hydrolysis was first performed on

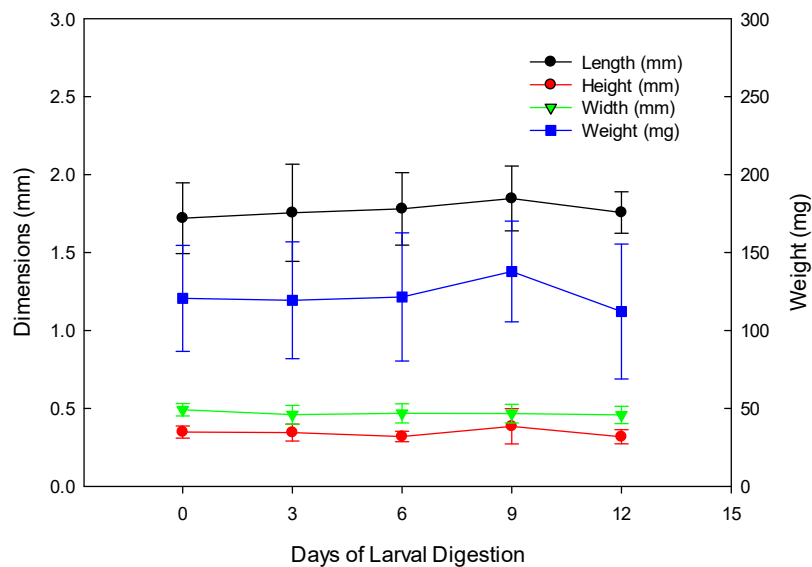
digested DDGS followed by enzymatic hydrolysis. This served the dual purpose of maximizing total glucose yields, as well as allowing for analysis of the cellulose degradation. The hypotheses were that SSF would improve the nutrient profile and digestibility of DDGS; that SSF would open up the DDGS component matrices making it more susceptible to enzymatic hydrolysis into glucose; and that BSFL digestion would degrade crystalline cellulose into amorphous cellulose making it more susceptible to acidic hydrolysis.

### 5.3.2 Evaluation of Larval Digestion of Lignocellulosic Biomass without Microbial

#### Pretreatment

This section shows the results of the ‘control SSF’ DDGS sample, and the effect of larval digestion on that sample. Control SSF is homogeneous ground DDGS which has been subjected to a sterile SSF treatment. This sample was made to evaluate the effect of larval digestion alone on 100% DDGS, a natural lignocellulosic biomass feed.

As seen on **Fig. 5-2**, the weight of larvae did not change much during the larval digestion period of the control DDGS. The dry substrate utilization of DDGS by larvae was 81% after



**Fig. 5-2 Larval Parameters in Control DDGS + Larvae**

Six larvae were randomly chosen and measured to check their growth. N = 6; means and std. dev.

twelve days, as shown on **Table 5-1**. This proved that the larvae can survive on DDGS as a sole feed source, and that they can efficiently reduce the dry matter of the substrate. If the larvae used

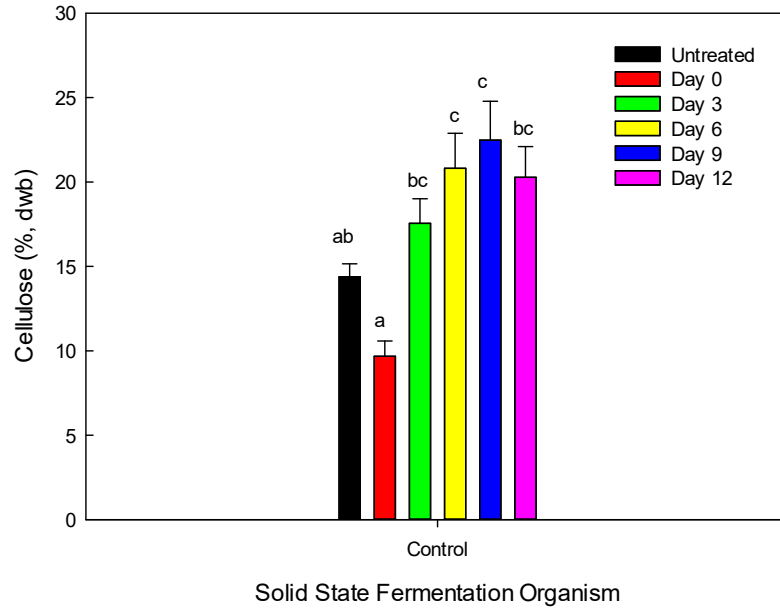
to inoculate the DDGS were young larvae in their 1<sup>st</sup> or 2<sup>nd</sup> instar instead of their 4<sup>th</sup> or 5<sup>th</sup> instar as was the case for this sample, then it is expected that an overall growth in larval biomass would be observed alongside the substrate utilization.

**Table 5-1 System Changes in Control DDGS + Larvae**

Day	0	3	6	9	12
% Substrate Utilized by Larvae (dwb)	0	29	44	75	81
Weight of Larvae Under Unchanging Numbers (mg, ww)	73.83	73.10	74.39	84.38	68.68

The cellulose content of DDGS, as seen on **Fig. 5-3**, saw a decrease following the 7 day “control SSF” period, i.e., DDGS treated with the same process as SSF DDGS, but without inoculation of SSF strains, shown by comparing the day 0 larval digestions to the untreated DDGS. This cellulose content change was unexpected. This change in cellulose content of DDGS was also observed to occur during month-long storage of sterilized DDGS at refrigerated temperatures (data not shown). We cannot disregard the untreated sample, as it is needed to form an understanding of the changes to DDGS during the SSF period, and provides insights towards the processing of DDGS. One insight is that the sample must be sufficiently saturated with moisture to attain accurate cellulose content measurements. The following is a brief explanation for the mechanism behind changes to observed cellulose contents during storage. Since no organism was present during this control SSF period, the loss in observed cellulose during that time is proposed to be due to an opening of the DDGS component matrices, especially lignocellulose and proteins. As the moisture penetrates the structure of DDGS over time, the components become more susceptible to acidic hydrolysis. The cellulose concentration assay, from which **Fig. 5-3** results are obtained, involved using strong acids to dissolve components except cellulose from DDGS. These non-cellulose components were then removed by washing the cellulose with solvents. Therefore, as the moisture penetrates the DDGS component matrices such as during the control SSF period, and they became more susceptible to acids, the true cellulose content was seen. The cellulose content is shown in **Fig. 5-3** to be 10% (dwb) of DDGS, which agrees very well with the literature value of 10% crude fiber. This softening and opening of the DDGS structure explains the observed decrease in cellulose content during the “control SSF” period. This speculation is supported by the acidic sugar liberation examination (**Fig. 5-6**), which showed that the amount of glucose released via dilute acid hydrolysis increased

after the control SSF period. Again, this is proposed here to be due to a penetration of moisture into the component DDGS matrices which increased their susceptibility towards acidic hydrolysis by an increased access of acids to the DDGS components. The cellulose content increased significantly over the twelve day larval digestion period. It indicated that the larvae can

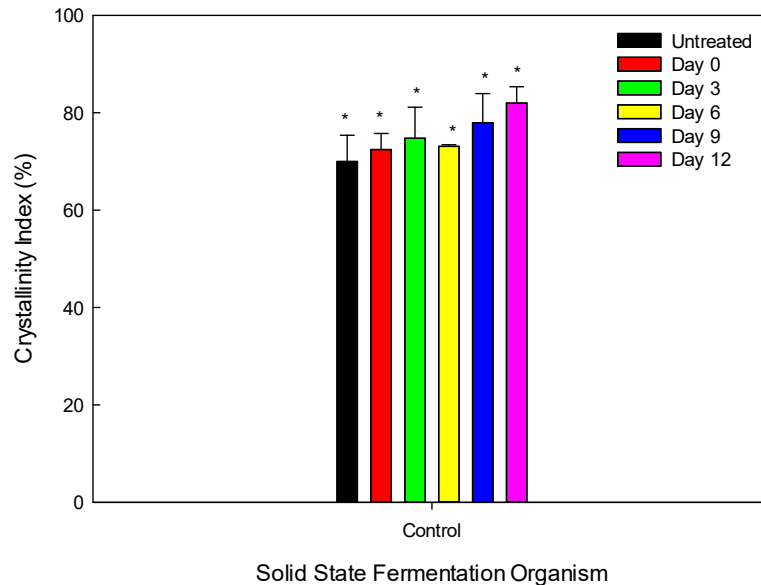


**Fig. 5-3 Cellulose Content of Control DDGS + Larvae during 12 Day Larval Digestion**

Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the control solid state fermentation (SSF) period which involved a sterile fermentation period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the control SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD;  $p < 0.001$ ; LSD = 2.90;

use non-cellulosic materials for their activities and left cellulose behind, increasing the cellulose concentrations.

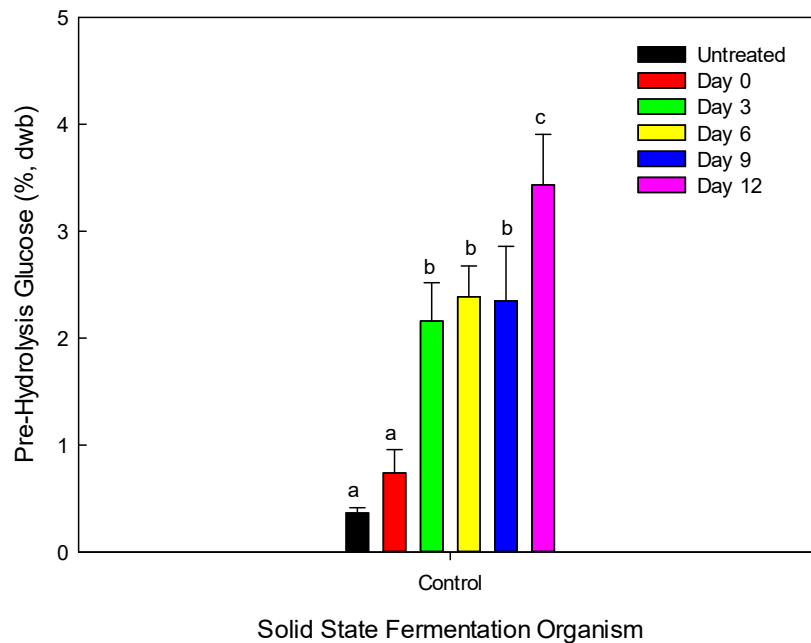
The crystallinity of the extracted cellulose, shown on **Fig. 5-4**, increased from both the control SSF treatment and larval digestion period although this change was not statistically significant.



**Fig. 5-4 Crystallinity Index of Cellulose from Control DDGS + Larvae during 12 Day Larval Digestion**

Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the control solid state fermentation (SSF) period which involved a sterile fermentation period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the control SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD; p = 0.0689; LSD = 9.74;

Free glucose in the substrate, shown on **Fig. 5-5**, increased from both the control SSF and from the larval digestion period. As previously mentioned, this sugar indicates the amount of glucose liberated during the SSF and BSFL treatments. After the larval digestion started on Day 0, the free sugar increased, indicating the free sugar was produced through the activities of larvae and associated gut microbial flora.

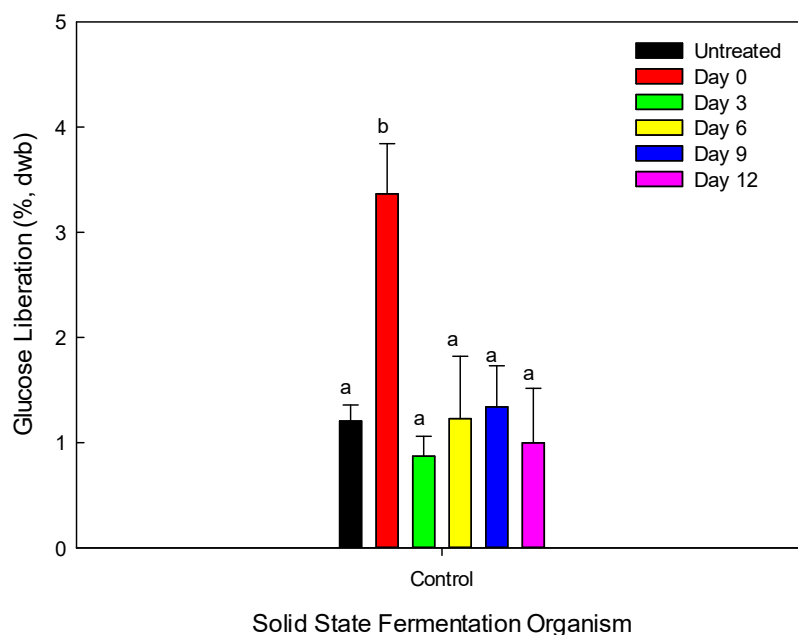


**Fig. 5-5 Free-Glucose in Spent Feed from Control DDGS + Larvae during 12 Day Larval Digestion**

Glucose % =  $[\text{mg glucose}/(\text{mg dry wt. of substrate} * 0.507)] * 100\%$ ; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Pre-hydrolysis or free-glucose is a measure of the soluble glucose concentration of a substrate. In DDGS, it is produced during larval digestion via enzymatic actions of the Black Soldier Fly larvae (BSFL), and via mechanical hydrolysis by the BSFL of bound sugars, starches, and amorphous cellulose. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the control solid state fermentation (SSF) period which involved a sterile fermentation period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the control SSF period, prior to larval inoculation. N = 6; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD;  $p < 0.001$ ; LSD = 0.440;

The sugar liberation of control DDGS via acidic hydrolysis is shown on **Fig. 5-6**. Acid hydrolysis sugar liberation of DDGS following the control SSF period, shown by comparing the untreated sample to day 0 on **Fig. 5-6**, increased significantly. The amount of liberated glucose decreased significantly by day three to similar levels to untreated DDGS and remained there through day 12 of larval digestion. The initial increase in acidic sugar liberation during the control SSF period would be due to the following: allowing a long moisture absorbance time swelled the starch, protein, and lignocellulose matrices giving rise to higher liberated glucose compared to untreated DDGS due to greater access of acids to glucans. Starch is more susceptible to hydrolysis than cellulose, is easily hydrolyzed by acid. It is present in DDGS, accounting for

up to 10% of the carbohydrates in DDGS, and gave rise in part to the large day 0 sugar liberation as compared to later sampling days. This starch was primarily utilized by the BSFL during the first three days of digestion and allowed for the response from less digestible substrate components, primarily cellulose, to be observed after day 0. In addition to potential starch response following the control SSF period, any native amorphous cellulose regions would remain after the control SSF period in the absence of SSF organisms' actions, and be hydrolyzed by the dilute acid. Acidic hydrolysis was also a measure of the amorphous cellulose.



**Fig. 5-6 Sugar Liberation by Acid Hydrolysis of Spent Feed from Control DDGS + Larvae during 12 Day Larval Digestion**

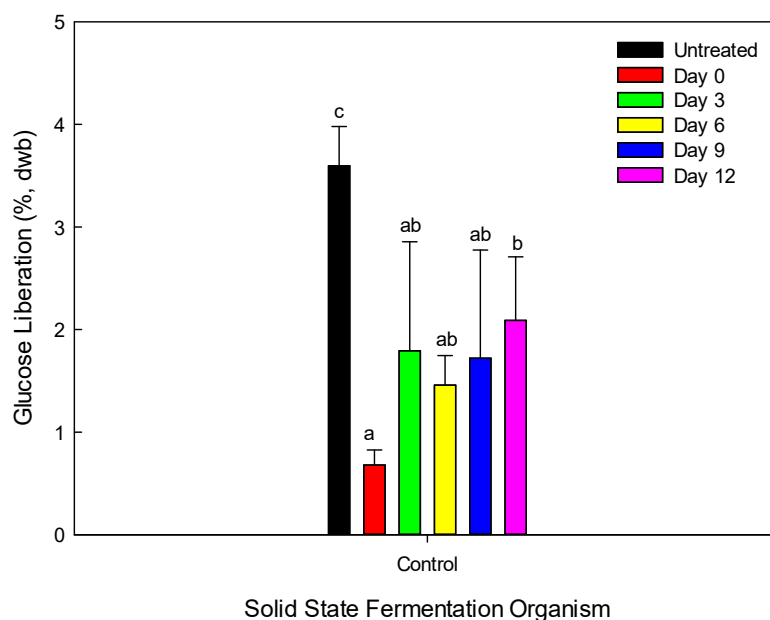
Glucose % = [mg glucose/(mg dry wt. of substrate \* 0.507)] \* 100%; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Glucose liberated from acid hydrolysis is a measure of the structurally weak glucose concentration of a substrate such as starches and amorphous cellulose. BSFL quickly digest starches. Longer larval digestion times therefore show primarily amorphous cellulose glucose. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the control solid state fermentation (SSF) period which involved a sterile fermentation period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the control SSF period, prior to larval inoculation. N = 6; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD;  $p < 0.001$ ; LSD = 0.73;

From **Fig. 5-5**, larval digestion increased the free-glucose in the control sample, indicating a removal and hydrolysis of amorphous cellulose to glucose. This correlated to the increase in CI shown on **Fig. 5-4** during that time. As the larvae digested the control sample, the



amorphous cellulose and other glucose containing substrates such as starch and glucose-protein complexes were digested by the larvae which explains why days 3-12 showed lower acidic hydrolysis glucose yields (i.e., less amorphous cellulose) compared to day 0. The acid hydrolysis trend from days three to twelve of the control sample closely follow that of the cellulose content of the substrate during that period.

Although the cellulose content increased from day 3 to day 12 of larval digestion, the acidic hydrolysis glucose yields remained relatively flat during that time period. Acidic hydrolysis yields are a measure of the amorphous cellulose in DDGS. This suggested that BSFL alone did not possess a strong ability to degrade cellulose structure from a crystalline to amorphous state alone, and confirms the crystallinity index results from **Fig. 5-4** which shows this to be the case.



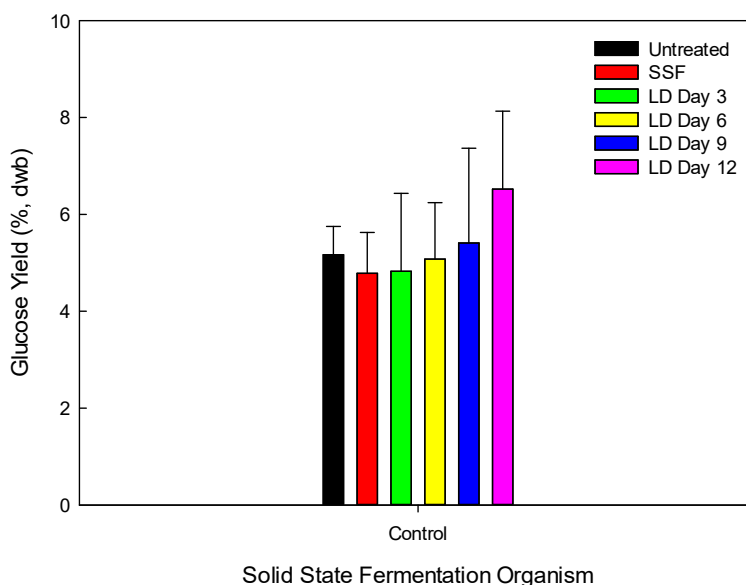
**Fig. 5-7 Sugar Liberation by Enzymatic Hydrolysis of Spent Feed from Control DDGS + Larvae during 12 Day Larval Digestion**

Glucose % =  $\text{mg glucose} / (\text{mg dry wt. of substrate} * 0.507) * 100\%$ ; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Enzymatic hydrolysis is preceded by dilute acid hydrolysis which removes structurally weak glucose polymers and free-glucose. Glucose liberation via enzymatic hydrolysis (cellulase) is therefore a measure of the amount of sugars which can be recovered from crystalline cellulose particles under the current system. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the control solid state fermentation (SSF) period which involved a sterile fermentation period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the control SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD;  $p > 0.006$ ; LSD = 1.23;

Several factors determine the total available sugar from DDGS such as the crystallinity index of the cellulose and the shift in relative composition of components in the substrate. Although changes in CI of the control SSF and larval digestion sample (**Fig. 5-4**) were insignificant, as the crystallinity index and concentration of the cellulose increased, so too did the glucose yield from enzymatic hydrolysis increase (**Fig. 5-7**). The enzymatic hydrolysis of the control SSF sample, shown on **Fig. 5-7**, showed a decrease after the control SSF period, and a significant increase to day three. The glucose liberation percent increased slightly thereafter, due to an increase in the concentration of cellulose in the undigested larval feed.

Untreated DDGS showed high enzymatic sugar liberation. Because the DDGS was not held for extended periods of time at high moisture, there was insufficient moisture penetration into the substrate which prevented the acidic hydrolysis from liberating as much glucose from the untreated sample as it otherwise would have. The glucose which escaped acid hydrolysis in the untreated sample instead became liberated during the enzymatic hydrolysis of the untreated sample. The enzymatic sugar liberation assay was done at slightly acid pH in a liquid sample suspension for 48 hours. These factors combined to release the starch glucoses similarly to day 0 acid hydrolysis as shown above. An increase in the enzymatic sugar liberation during larval digestion correlated with an increase in the total cellulose content of the undigested larval feed, and may indicate that the larvae have increased the crystalline cellulose's susceptibility to enzymatic hydrolysis. This was likely due to a micronization of the DDGS fibers and an increase in their surface area. Easily hydrolyzed glucose polymers and amorphous cellulose was removed during acid hydrolysis performed prior to this assay.

The total sugar yield, shown on **Fig. 5-8**, from the process increased during the larval digestion time, as well as from untreated to day 12 of larval digestion. Increases to the free glucose in the feed bed were the largest contributor to the increases in the total sugar yields from DDGS.



**Figure 5-8 Combined Sugar Liberation of Spent Feed from Control DDGS+Larvae during 12 Day Larval Digestion**

Glucose % =  $\text{mg glucose} / (\text{mg dry wt. of substrate} * 0.507) * 100\%$ ; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Combined sugar liberation is a combination of the free-glucose, and yields from both acidic and enzymatic hydrolysis of glucose. Error bars are added standard deviations from each analysis. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the solid state fermentation (SSF) period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation.

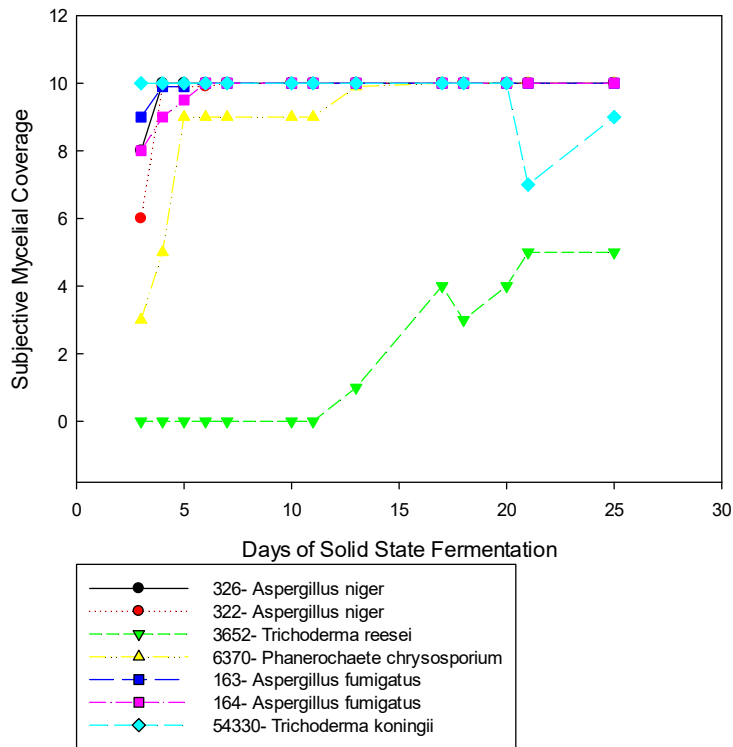
Overall, data from BSFL digestion of DDGS without SSF indicated that larval digestion can efficiently concentrate cellulose in DDGS, but it did not necessarily increase the ratio of amorphous to crystalline cellulose. Some of the amorphous cellulose was hydrolyzed and digested along with starches during BSFL digestion. The removed amorphous cellulose and starch glucose was not all utilized by the larvae, as an increase in free glucose in the substrate was seen over time. Larval digestion increased the surface area of cellulose which, along with increasing the cellulose concentration, increases the enzymatic sugar liberation. Overall this contributed to a slight increase in glucose yields by day 12 of larval digestion of DDGS

compared to the untreated sample (**Fig 5-8**). It suggested that the larval digestion can contribute to the liberation of sugar, however, the larval treatment requires additional means to increase the efficiency of sugar liberation to a level utilizable in the larger production scheme. The addition of enzymes through SSF, or through simple addition of commercial enzymes is needed to increase the amount of sugars which can be liberated from DDGS after larval digestion. The treatment of SSF was considered as the pretreatment of DDGS in the following experiments to modify DDGS before larval treatment.

### **5.3.3 Solid State Fermentation and Larval Digestion of Lignocellulosic Biomass**

#### **5.3.3.1 Evaluation of Fungal Organism and Particle Size Effect on Lignocellulosic Biomass**

Given the ability of the larvae to increase the relative cellulose content while removing the other constituents from DDGS, and the ability of SSF organisms to degrade the cellulose, it was proposed to combine the two treatments by doing larval digestion first, followed by SSF. Previous literature had indicated the presence of anti-microbial compounds capable of disrupting bacterial and fungal species growth. This was also supported by the egg laying buckets in our BSFL rearing. The substrate typically became covered in mold, and then when the BSF eggs hatched, the neonate larvae would digest the substrate and mix it about. The mold would no longer be visible at that point. Thus the order of SSF and larval treatments may be critical. To narrow down the list of fungal organisms suitable for SSF of DDGS, a series of experiments were made and are reported here. The examined conditions included the particle size of the starting DDGS material (achieved through knife milling, and separation by sieving of wheat DDGS), SSF microorganism used, acidic hydrolysis conditions, order of SSF/larval addition, and starting size of the larvae.



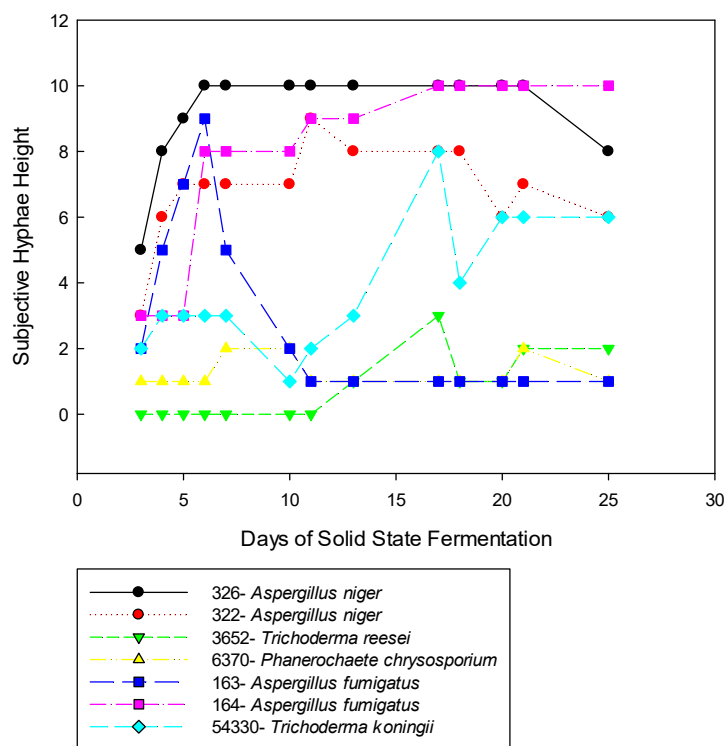
**Fig. 5-9 Mycelium Coverage of DDGS (0-10)**

Subjective mycelia coverage is a measure of the cultivation of dried distiller's grains with solubles (DDGS) via fungal organisms as determined by visual inspection of the DDGS surface covered by fungal mycelium. The scale went from 0 (no coverage) to 10 (full coverage).

**Fig. 5-9** shows the values for the subjective measurement of mycelium coverage of DDGS over time. Most strains had an extensive mycelia network by the 5<sup>th</sup> day post-inoculation, except for *Trichoderma reesei*, which showed very little mycelia growth. **Fig. 5-10** shows the height at which the fungal growth protruded from the surface of the DDGS. The height of the fungi increased for most isolates, except for *Aspergillus fumigatus* which peaked quickly then receded, and *Phanerochaete chrysosporium* which remained steady. This growth parameter was measured as an indicator of the virility of the fungi, and its suitability to utilize DDGS as a growth substrate however these growth patterns were later revealed to be more symptomatic of the fungal strain used, rather than an indicator of the strain's growth. Different organisms have different growth patterns, and the enzymatic contributions or substrate degradations cannot be

accurately predicted from visual inspection alone. However, these measurements may serve as a guide for additional fermentations by these organisms within the same or similar systems.

After the SSF run of DDGS, done to determine which SSF organisms were the most suitable to break down DDGS for subsequent larval digestion, the DDGS was subjected to dilute acid and subsequent enzymatic hydrolysis to quantify the amount of overall substrate and cellulose degradation.



**Fig. 5-10 Protruding Hyphae Height (0-10)**

Subjective hyphae height is a measure of the cultivation of dried distiller's grains with solubles (DDGS) via fungal organisms as determined by visual inspection of the volume of container occupied by fungal hyphae. The scale went from 0 (no hyphae) to 10 (expansion of hyphae to full container volume).

As shown on **Table 5-2**, the highest rate of acidic sugar liberation was seen by *Aspergillus fumigatus* BRRL 163, and *Aspergillus niger* BRRL 326. Given that the sugar liberation for enzymatic hydrolysis was fairly consistent across the samples, the combined values of sugar liberation for both acidic and enzymatic hydrolysis were nearly identical to those of the acidic sugar liberation, albeit slightly higher. The sugar liberation values shown below are much less than for control DDGS + larvae. The sugars below are total reducing sugars as measured by

3,5-DNS, which was later found to be sub-optimal for measuring sugars in acidic solutions. All other sugar measurements pertaining to SSF were obtained using a glucose specific enzymatic assay which had a larger pH tolerance, and performed well in our system. Based on the results of the sugar liberation and growth patterns of the SSF fungi, *Aspergillus niger*, *Trichoderma koningii*, *Phanerochaete chrysosporium*, and *Aspergillus fumigatus* were chosen for further use in SSF combined with larval digestion of DDGS.

**Table 5-2 Sugar Liberation (% , reducing sugar mgs/ dry weight of the carbohydrates in DDGS) of Post-SSF DDGS to Reducing Sugars by Acidic and Enzymatic Hydrolysis**

Fungal Identity	Sample	Sugar Liberation (%)	Sugar Liberation (%)
		DDGS to Sugars by Acidic Hydrolysis	DDGS to Sugars by Enzymatic Hydrolysis
<i>Aspergillus niger</i>	BRRL 326	2.67	1.14
<i>Aspergillus niger</i>	BRRL 322	1.47	1.21
<i>Trichoderma reesei</i>	BRRL 3652	1.31	1.35
<i>Phanerochaete chrysosporium</i>	BRRL 6370	2.06	1.22
<i>Aspergillus fumigatus</i>	BRRL 163	2.69	1.37
<i>Trichoderma koningii</i>	BRRL 54330	1.93	1.40
<i>Aspergillus fumigatus</i>	BRRL 164	1.91	1.09

It was observed that the proposed fungal strains could grow on DDGS, and they could increase the degradation of the substrate compared to (non-fermented) control DDGS. However, the fungal growth was very slow, and the amount of substrate degradation was low. During these experiments, we found that the availability of oxygen affected the growth of fungi in our setup. Therefore containers which had holes in the lid were used in subsequent SSF applications. The substrate was mixed each day during SSF aseptically.

As mentioned earlier, mold growth may be inhibited by the secretion of BSFL. A previous research, shown on **Table 5-3**, has shown an antibiotic activity from larval secretions (waste) as well as from homogenized larval bodies (representing an internal immune system for the insect, thought to be present primarily in the haemolymph). One of the antimicrobial substances in larval extracts has been identified as hexanedioic acid<sup>188</sup>.

**Table 5-3 Minimum Inhibitory Concentration of the Larval Extract Fractions from *Hermetia illucens* larvae**

Microorganisms	Strain	MIC of the extract fractions (mg/mL)			MIC of antibiotics (µg/mL)	
		Water	Ethyl acetate	Chloroform	Methicillin	Ampicillin
Gram-positive bacteria						
MRSA† (clinically isolated)		25	NT‡	>100	>80	>80
<i>Staphylococcus aureus</i>	KCCM 40881	>100	>100	>100	80	2.5
<i>S. aureus</i>	KCCM 12256	100	NT	>100	2.5	2
<i>S. epidermidis</i>	KCCM 35494	50	25	>100	>20	10
<i>Kocuria rhizophila</i>	KCCM 11236	25	NT	>100	<0.3125	<0.3125
<i>Micrococcus luteus</i>	KCCM 11326	25	NT	>100	NT	NT
<i>Bacillus subtilis</i>	KCCM 11316	12.5	25	>100	0.078125	0.078125
Gram-negative bacteria						
<i>Escherichia coli</i>	KCCM 11234	12.5	>100	>100	>80	20
<i>Enterobacter aerogenes</i>	KCCM 12177	25	NT	>100	>20	>20
<i>Pseudomonas aeruginosa</i>	KCCM 11328	12.5	25	>100	NT	NT
Yeast						
<i>Candida albicans</i>	KCCM 11282	25	50	>100	>20	>20

†MRSA, methicillin-resistant *Staphylococcus aureus*.

‡NT, not tested.

Reprinted from Park *et al.*<sup>213</sup>, with permission from Wiley.

To test the feasibility larval digestion followed by SSF, spent feed which had been stored in the freezer was placed into 250mL Erlenmeyer flasks. Half of the feed was sterilized via autoclave at 121°C for 15 minutes, thinking that the inhibitory substance may be heat labile. Three-day seed cultures of the SSF organisms were used to inoculate the spent feed. The flasks were stored at 30°C and checked daily for the presence of fungal growth. After a week of incubation, growth was seen on the sterilized spent feed by *Aspergillus fumigatus*. The growth was concentrated in areas of low feed concentration, not colonizing the large chunk of substrate directly. It is possible that the other fungal strains under analysis were growing, but just were not detected by visual inspection. For all organisms, observed or not, the spent feed was a poor substrate for fungal growth and seemed to retard the metabolic activities of the fungi.

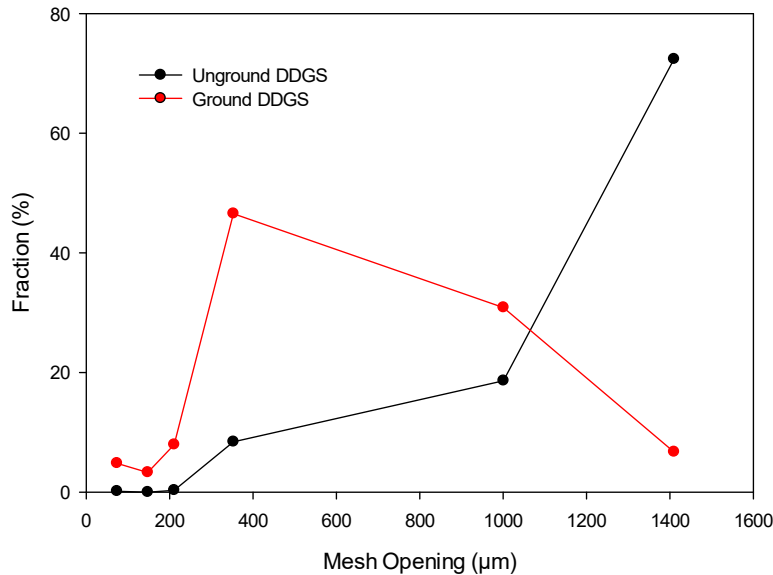
Several fungal organisms were used in SSF of DDGS, as well as *Lactobacillus plantarum* isolated from digested feed beds of the BSFL which was discussed in Study 1 presented here. *Phanerochaete chrysosporium* is a white-rot fungi which secrete of a variety of peroxidases and oxidases which act non-specifically on the heteropolymer lignin through the generation of lignin free-radicals, which then undergo cleavage reactions<sup>83</sup>. *Phanerochaete chrysosporium* is unique among white rot fungi in that it leaves cellulose nearly unchanged while degrading other constituents. *Phanerochaete chrysosporium* is typically known for its ligninolytic abilities; however the fungus can produce both hemicellulases (xylanase and β-xylosidase) and cellulases



(CMCase and  $\beta$ -glucosidase). It also has a high optimum temperature of 40 °C which makes it ideal for compost piles in which heat transfer may be limited. These characteristics have made *Phanerochaete chrysosporium* a candidate for use in biotechnology applications related to cellulosic materials. *Aspergillus fumigatus* is a saprotroph which is fairly ubiquitous throughout nature, and is often found in decaying matter, such as in compost heaps<sup>214</sup>. It produces a diverse variety of extracellular enzymes including cellulases and other glycoside hydrolases, amylases, hemicellulases, lignin degrading enzymes, poly-peptide degrading enzymes, chitinases, lipases, and phosphatases which are useful bioprocessing of lignocellulosic materials. *Trichoderma koningii* is another fungal strain which is ubiquitous throughout nature, being frequently isolated from soil samples. It produces a variety of extracellular enzymes, but is most frequently noted for its cellulase enzyme production. One research group isolated and purified four principle components of cellulase from *Trichoderma koningii*, and all were found to be able to hydrolyze various modified cellulose polymers, albeit at different rates<sup>215</sup>. Comparatively little research has been conducted on *Trichoderma koningii* as compared to other species in the *Trichoderma* genus. However among those *Trichoderma* species for which a larger body of research is available, a variety of extracellular enzymes are noted which suggests, by extension, that this would be true for *Trichoderma koningii* as well. *Aspergillus niger* is a common contaminant of food and produces black mold on some fruits and vegetables. It has GRAS status by the U. S. FDA under the Food, Drug, and Cosmetic Act<sup>196</sup>. It produces a wide variety of extracellular enzymes, and thus is used in a wide variety of industrial processes such as: the production of glucoamylase for sugar production; pectinase production used for juice and wine clarification; production of proteases which are used to reduce the gluten content of beer; and more. Relevant to this project is the production of cellulase complexes. *Aspergillus* is known to produce high amounts of  $\beta$ -amylase particularly<sup>82</sup>.

Another question which was investigated at this time was whether the particle size of the DDGS had an effect on its degradation by SSF organisms, and subsequently, by larvae. Homogeneous, fine, and coarse samples of DDGS were moistened and sterilized for use in SSF. Four strains (*Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus fumigatus*, *Aspergillus niger*) were selected which showed the greatest substrate degradation. Urea was also added to the sterilized DDGS, which was adjusted to 75% moisture prior to the addition of fungal seed culture.

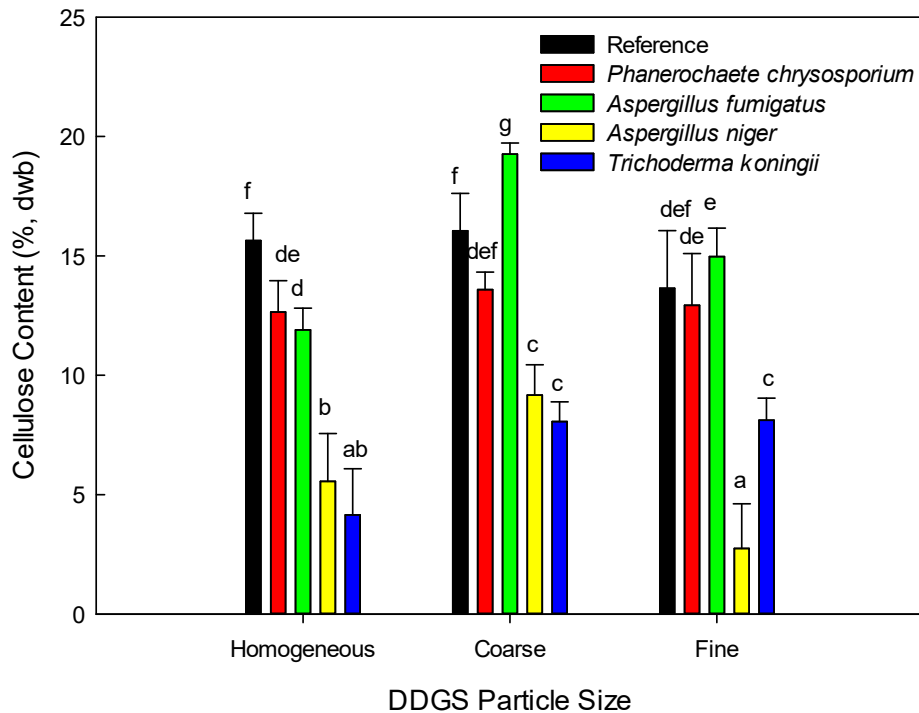
From **Fig. 5-11**, it is seen that greater than 72% by weight of unground DDGS has particle sizes greater than 1410 $\mu\text{m}$ . Milling of the DDGS had the effect of redistributing these larger fractions into smaller pieces, primarily greater than 353 $\mu\text{m}$ .



**Fig. 5-11 Particle Size Analysis of DDGS**

Grinding of dried distiller's grains with solubles was done using a knife mill. Particle size analysis was obtained using standard sieves. The fraction (%) represents the fraction of particles in the sample greater than the mesh size at that coordinate, but not greater than the next largest mesh size shown.

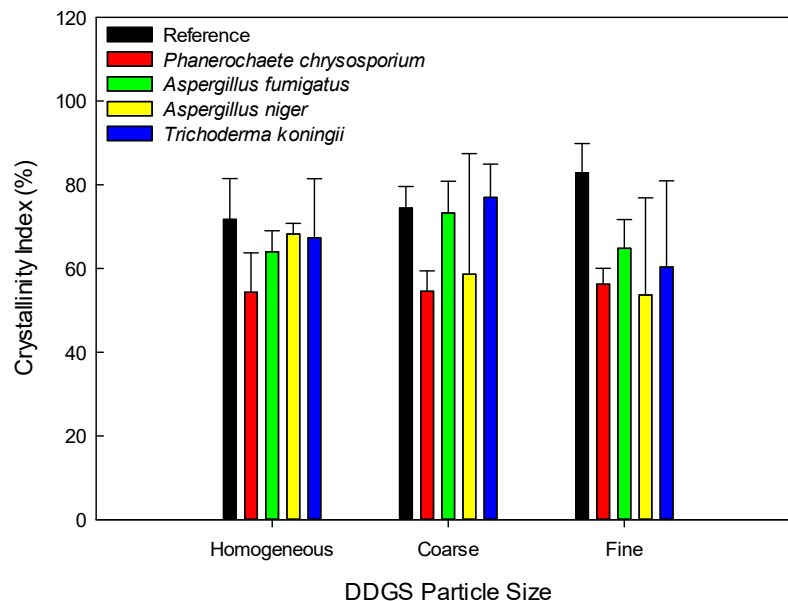
**Fig. 5-12** shows the cellulose content of ground DDGS following SSF. *Aspergillus fumigatus* resulted in the least decrease in cellulose content, followed by *Phanerochaete chrysosporium*, *Trichoderma koningii*, and *Aspergillus niger*. *Aspergillus niger* growing on the fine particle size, and *Trichoderma koningii* and *Aspergillus niger* growing on the homogeneous particle size showed the greatest reduction in cellulose content of all strain and particle size combinations.



**Fig. 5-12 Cellulose Content of Ground DDGS Fractions (dwb)**

Reference dried distiller's grains with solubles (DDGS) is an autoclaved sample of DDGS at the same particle size as the grouping, but without fermentation. N = 3; Means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD across all particle sizes;  $p < 0.001$ ; LSD = 2.49;

For all strains, except *Aspergillus niger* which saw the greatest cellulose % reduction in the fine particle size sample, the homogeneous blend showed the greatest reduction in cellulose content (**Fig. 5-12**). By comparing the cellulose content of fermented DDGS **Fig. 5-12** to the cellulase activities of the SSF organism **Fig. 5-15** we can see that higher cellulase activity leads to a lower cellulose content as would be expected. There appear to be some correlation between the CI of fermented DDGS cellulose (**Fig. 5-13**), and cellulase activity, but this relationship is less clear than that between cellulase and overall reductions in cellulose concentrations. As previously stated, the SSF organisms are known to produce different amounts of specific cellulases. *A. niger* for instance produces high amounts of  $\beta$ -glucosidase which cleaves two  $\beta$ -1,4 linked glucose monomers into glucose which alone wouldn't significantly reduce the crystallinity index. Additionally, these SSF organisms produce synergistic enzyme cocktails so predictions of changes to cellulose CI based on a broad cellulase assay are challenging. The



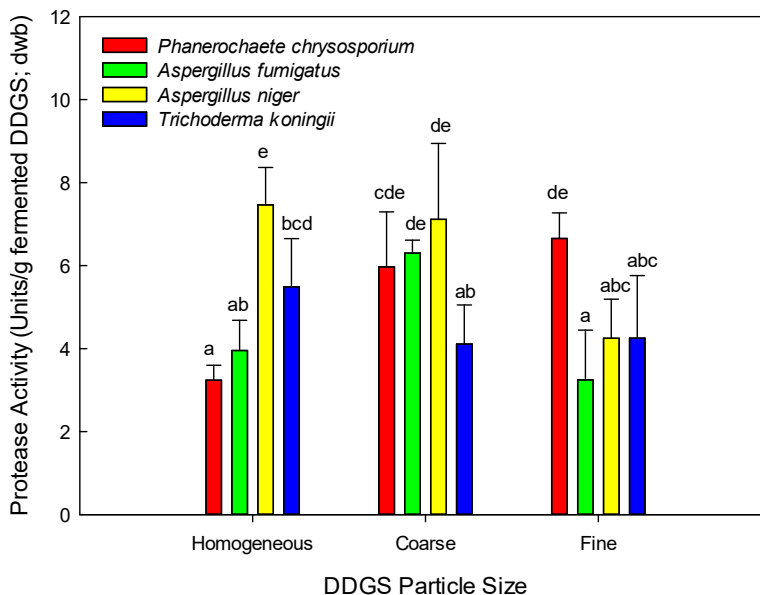
**Fig. 5-13 Crystallinity Index of Ground DDGS Cellulose**

Reference dried distiller's grains with solubles (DDGS) is an autoclaved sample of DDGS at the same particle size as the grouping, but without fermentation. N = 3; Not significant at  $\alpha=0.05$ ;  $p = 0.41$ ;

superior cellulase production for the homogeneous particle size among SSF organisms is thought to be due to the presence of large and small particles, which provide scaffolding for hyphae and quickly hydrolyzed source of nutrients respectively. For all strains and particle sizes of substrate, except *Trichoderma koningii* coarse ground DDGS, SSF reduced the crystallinity of the cellulose (Fig. 5-13). This makes sense as the extracellular enzymes produced by the SSF organisms serve to break down/apart the crystalline structure of the cellulose particles. Homogeneous ground DDGS samples had intermediary crystallinity index values; coarse ground DDGS had the highest crystallinity; and fine ground DDGS had the least. This supports the theory of extracellular enzyme degradation as the crystallinity index of cellulose is inversely correlated with the surface area of the particles. Smaller particles allow for the greatest access of enzymes to cellulose in the substrate.

Since the two main components in wheat DDGS are protein (40%) and carbohydrates (50%), the fermented DDGS fractions were tested for cellulase and protease. Most of the carbohydrates are assumed to be lignocellulose and resistant starch, with the former being the most prevalent. In order to test the enzyme activities of the post-SSF samples, 5g (wet wt.) of

sample was mixed into 25mL of 50mM Tris-HCl pH 7.5 with 100mM NaCl, vortexed and centrifuged, and the supernatant was decanted and stored. 5mL of buffer was added to the pellet which was again vortexed and centrifuged. The supernatant fractions were combined and stored at 4 °C. The enzyme activities were back calculated to represent a dry basis for the feeds used.



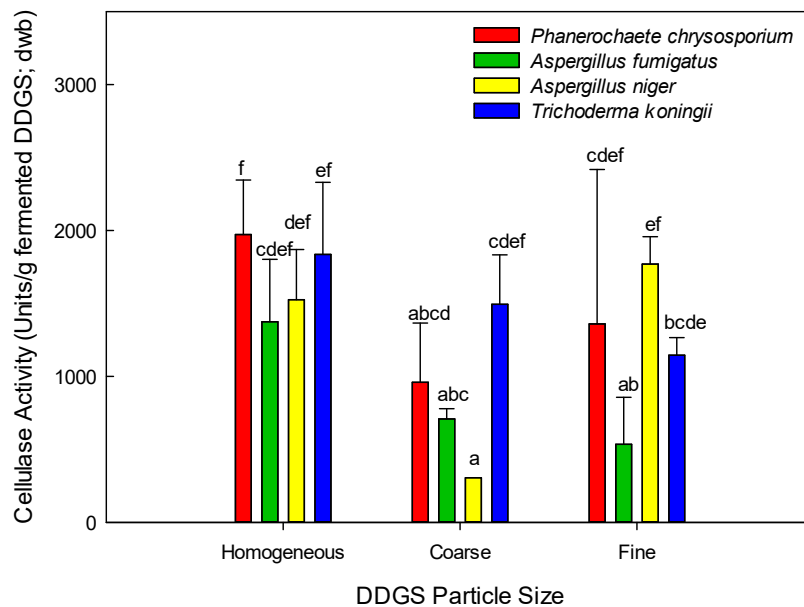
**Fig. 5-14 Protease Activity of Post-SSF Homogeneous, Coarse, and Fine DDGS Fractions**

N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD;  $p < 0.001$ ; LSD = 1.81;

The SSF resulted in protease production for all samples as summarized on **Fig. 5-14**. No trend was seen for the effect of particle size on the protease production by SSF organisms. *Aspergillus niger* was the best protease producer, and *Aspergillus fumigatus* the worst. The following discussion on **Figs. 5-14 to 5-21** will refer to individual samples by referencing the SSF organism followed by the ground DDGS particle size. So *Aspergillus niger* Coarse refers to SSF of coarse ground DDGS by the fungus *Aspergillus niger*. The relationship between protease activity and substrate particle size was less consistent across strains (**Fig. 5-14**). *Phanerochaete chrysosporium* showed the greatest protease activity with fine substrate size; *Aspergillus fumigatus* showed the greatest with coarse; *Aspergillus niger* showed the greatest with coarse, but homogeneous was a very close 2<sup>nd</sup>; and *Trichoderma koningii* showed the greatest with homogeneous particle size. *Aspergillus niger* Coarse was the greatest protease producer, followed

by *Aspergillus niger* Homogeneous/ *Trichoderma koningii* Homogeneous/ *Aspergillus fumigatus* Coarse/ *Phanerochaete chrysosporium* Fine which all tied for 2<sup>nd</sup> place essentially. Since the relationship between particle size and protease activity was inconsistent across strains, this suggests that the differences may be due to differences in strain metabolic activities.

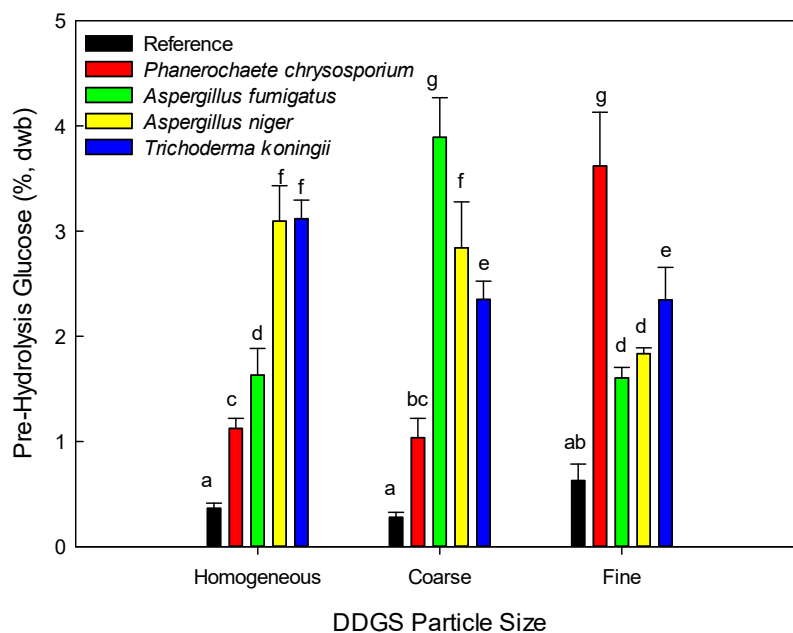
**Fig. 5-15** shows the cellulase activities of selected fungal strains used to treat ground DDGS fractions. Cellulase activity was the greatest across all strains, except for *Aspergillus niger* in which the fine particle size resulted in the highest cellulase activity, for the homogeneous particle size. *Phanerochaete chrysosporium* was a consistently good cellulase producer. *Aspergillus niger* and *Trichoderma koningii* were second to *Phanerochaete chrysosporium* in cellulase production, but differed on the particle size to which they responded best. *Aspergillus fumigatus* was the worst cellulase producer.



**Fig. 5-15 Cellulase Activity of Post-SSF DDGS**

N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD;  $p < 0.001$ ; LSD = 802.18;

Pre-hydrolysis glucose was increased by SSF for all samples as shown on **Fig. 5-16**. Extracellular enzyme production was hydrolyzing starch and cellulose faster than the fungi could uptake the glucose produced. When evaluating the effect of enzyme production by SSF



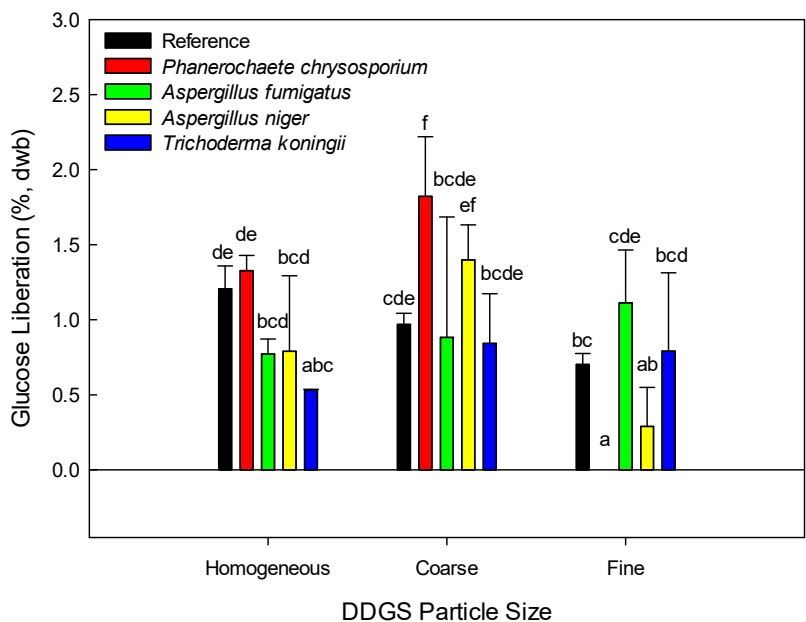
**Fig. 5-16 Pre-Hydrolysis Sugars from Post-SSF of Homogeneous, Course, and Fine DDGS Fractions**

Glucose % = [mg glucose/(mg dry wt. of substrate \* 0.507)] \* 100%; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Pre-hydrolysis or free-glucose is a measure of the soluble glucose concentration of a substrate. In DDGS, it is produced during solid state fermentation (SSF) via enzymatic actions of the SSF organisms. Reference dried distiller's grains with solubles (DDGS) is an autoclaved sample of DDGS at the same particle size as the grouping, but without fermentation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD;  $p < 0.001$ ; LSD = 0.43;

organisms towards sugar production, it is seen that the differences in pre-hydrolysis glucose (**Fig. 5-16**) follow closely the trend for differences in protease production (**Fig. 5-14**). Cellulase production wasn't correlated to pre-hydrolysis glucose. This makes sense as proteins comprise a much higher percentage of DDGS than cellulose. The degradation of proteins has a large impact on the release of sugars upon submerging of the substrate in liquid in liquid due to an opening of the DDGS protein matrix. Because the homogeneous ground DDGS particle size resulted in the greatest protease and cellulase production in general, the pre-hydrolysis glucose concentration was the greatest for that particle size among SSF organisms. In the case of *P. chrysosporium* and

*A. niger* the homogeneous particle size was second in pre-hydrolysis glucose to the fine and coarse particle sizes respectively (Fig. 5-16). This is because protease production was greatest for the *P. chrysosporium* Fine and *A. niger* Coarse samples (Fig. 5-14). In every sample, the free-glucose was higher following SSF than in the untreated DDGS due to the enzymatic activity of the SSF organisms. In summary, differences in the free-glucose in DDGS can be explained primarily by differences in protease activity of the fungal SSF organism.

The grinding process did not significantly change the amount of glucose which can be liberated from DDGS via dilute acid hydrolysis as shown by comparing the reference samples on Fig. 5-17. SSF by *Phanerochaete chrysosporium* on coarse DDGS significantly increased the amount of sugars which can be liberated by acid hydrolysis. *Phanerochaete chrysosporium* also showed the highest cellulase activity among SSF strains assayed, and shows excellent



**Fig. 5-17 Acid Hydrolysis Sugar Liberation from Post-SSF of Homogeneous, Coarse, and Fine DDGS Fractions**

Glucose % = [mg glucose/(mg dry wt. of substrate \* 0.507)] \* 100%; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Glucose liberated from acid hydrolysis is a measure of the structurally weak glucose concentration of a substrate such as starches and amorphous cellulose. Reference dried distiller's grains with solubles (DDGS) is an autoclaved sample of DDGS at the same particle size as the grouping, but without fermentation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD;  $p = 0.002$ ; LSD = 0.601;



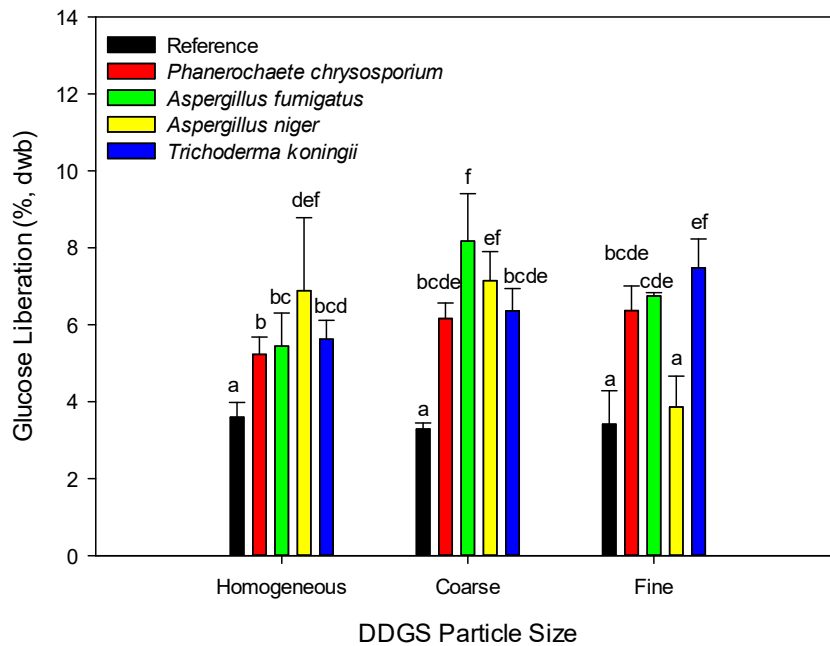
degradation of DDGS as determined by visual inspection. *Phanerochaete chrysosporium* is a white rot fungus which is well known for its cellulolytic ability. Extracellular enzymes produced by this organism degrade the structure of cellulose as well as the component matrices in the substrate which allow for a greater degree of substrate hydrolysis by dilute HCl.

By comparing the CI of fermented DDGS on **Fig. 5-13** to the sugars released by acidic hydrolysis on **Fig. 5-17** we can see that they share an inverse relationship for the homogeneous and coarse DDGS particle sizes. As SSF reduces the crystallinity of cellulose, converting crystalline cellulose to amorphous cellulose, these amorphous celluloses are hydrolyzed during acidic hydrolysis to produce higher glucose yields. There is also a strong correlation between the amount of sugars released by acidic hydrolysis (**Fig. 5-17**) and the cellulose content of DDGS following SSF (**Fig. 5-12**) as was expected.

SSF did not significantly increase the amount of amorphous cellulose in DDGS evidenced by the small change in acidic hydrolysis glucose yields compared to the reference for every sample except *P. chrysosporium* Coarse. This response may be due to a hydrolysis of starch polymers during the SSF period as well, which would also further explain the large increases seen in pre-hydrolysis glucose concentrations.

**Fig. 5-18** shows that *Aspergillus niger* and *Trichoderma koningii* resulted in the greatest sugar liberation by enzymatic hydrolysis. Enzymatic hydrolysis accounted for a large amount of the total sugar yield from DDGS hydrolysis. Therefore, while factors prevent the increase in sugar yield from acid hydrolysis, changes to DDGS structure enabled large increase enzymatic sugar liberation yields. As expected, the release of glucose from enzymatic hydrolysis (**Fig. 5-18**) was correlated strongly to the cellulose concentration of DDGS (**Fig. 5-12**) with higher cellulose concentrations producing higher glucose yields. Enzymatic hydrolysis glucose yields also increased as the CI of fermented DDGS cellulose (**Fig. 5-13**) increased. There was more crystalline cellulose in the samples with high cellulose concentrations with high CI. This led to greater glucose yields from enzymatic hydrolysis of those samples. Both the cellulose content, and CI of the samples share an inverse relationship with the cellulase activity in those samples. Therefore, cellulase activities in fermented DDGS (**Fig. 5-15**) had an inverse relationship to glucose yields via enzymatic hydrolysis (**Fig. 5-18**) in SSF DDGS.

Surprisingly, given the superior enzyme production by the homogeneous ground DDGS particle size, this size yielded the least total glucose from fermented DDGS (**Fig. 5-19**), except in the case of *A. niger* when it was second after the coarse particle size. The coarse particle size yielded the most glucose due to inferior degradation of DDGS by the SSF organisms. A lack of SSF action resulted in more amorphous being present on the coarse ground DDGS resulting in higher acidic hydrolysis glucose yields than other particle sizes. On **Fig. 5-12** it is seen that the coarse particle size had the highest cellulose content which led to the greatest enzymatic hydrolysis glucose yields (**Fig. 5-18**). Without considering bioconversion of proteins to larval biomass, a coarse DDGS particle size is best for glucose production, but the worst for efficiency



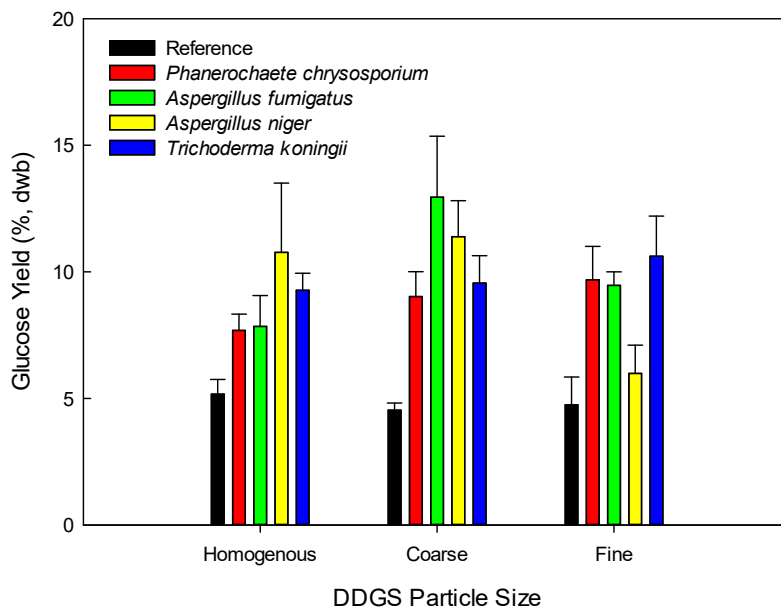
**Fig. 5-18 Enzymatic Hydrolysis Sugar Liberation from Post-SSF of Homogeneous, Coarse, and Fine DDGS Fractions**

Glucose % = [mg glucose/(mg dry wt. of substrate \* 0.507)] \* 100%; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Enzymatic hydrolysis is preceded by dilute acid hydrolysis which removes structurally weak glucose polymers and free-glucose. Glucose liberation via enzymatic hydrolysis (cellulase) is therefore a measure of the amount of sugars which can be recovered from crystalline cellulose particles under the current system. Reference dried distiller's grains with solubles (DDGS) is an autoclaved sample of DDGS at the same particle size as the grouping, but without fermentation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD;  $p < 0.001$ ; LSD = 1.357;

of cellulose conversion to glucose.

The fine DDGS particle size, which had the second highest cellulose content following SSF, yielded the second highest total glucose yields for most organisms (**Fig. 5-19**). Therefore it was the second most efficient for conversion of cellulose into glucose. These sugars were derived from high pre-hydrolysis glucose, and enzymatic hydrolysis. These are both factors of having a small particle size which limited the effect of protein matrices binding proteins, as well as allowed access for enzymes during enzymatic hydrolysis.

The homogeneous particle size resulted in the lowest total glucose production among the particle sizes (**Fig. 5-19**). For all organisms except *A. fumigatus* this difference was under two percent from the highest yielding particle size for a given organism indicating that it wasn't extremely lower yielding. Because the homogeneous particle size also had the lowest cellulose content as shown on **Fig. 5-12**, but resulted in nearly comparable total glucose yields it was the most efficient conversion of cellulose to glucose. SSF increased the total glucose yield compared



**Fig. 5-19 Combined Sugar Liberation from Post-SSF of Homogeneous, Coarse, and Fine DDGS Fractions**

Glucose % =  $\text{mg glucose} / (\text{mg dry wt. of substrate} * 0.507) * 100\%$ ; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Combined sugar liberation is a combination of the free-glucose, and yields from both acidic and enzymatic hydrolysis of glucose. Error bars are added standard deviations from each analysis.

to untreated DDGS in every case. Based on the above information, it was decided to utilize the homogenous blend DDGS in a combination experiment of SSF and larval digestion. This has the added benefit of reducing the amount of grinding needed. It also confirms that particle size reduction to a point can increase the production of extracellular enzymes of interest. The homogeneous ground DDGS sample was chosen for all further SSF work, including the control DDGS + Larvae shown in the previous section.

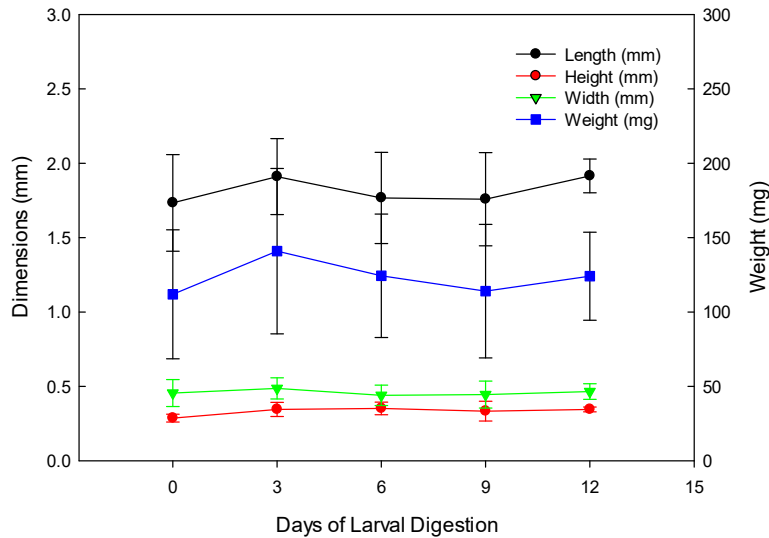
In summation of the above results it was seen that: DDGS is a suitable organism for robust fungal growth; the selected fungal strains have high protease and cellulase activity which act upon the substrate during SSF; SSF decreases the cellulose content and CI of cellulose of DDGS through cellulase production; free-glucose in DDGS is highly correlated to protease activity following SSF; that SSF does not increase the amount of glucose which can be liberated by acid hydrolysis from DDGS due to a reduction in overall cellulose content and uptake by fungal organisms; and that SSF greatly increases enzymatic glucose liberation, and free-glucose in the substrate which greatly increase total glucose yields from DDGS compared to untreated DDGS.

### **5.3.3.2 Larval Digestion of SSF-Treated Lignocellulosic Biomass**

From this point on, the results will be referring to a combination of SSF of homogeneous ground DDGS. This fermented DDGS was fed to larvae in order to assess the effects of SSF on DDGS, and how SSF contributes to larval digestion of DDGS.

From the SSF combined with BSFL digestion experiments it was shown that BSFL are capable of growing on post-SSF DDGS. As can be seen from **Figs. 5-20 to 5-24**, the wet weight of the larvae increased for all samples except for *Lactobacillus plantarum*, but the gain was not identical across SSF types. Additionally, the weight of the larvae fluctuated over the digestion period, and did not show a linear increase across time. One explanation for this is that the larvae gained weight initially as the nutrients in the substrate were converted into larval biomass. Towards the beginning of the larval digestion period the easily digested components of the substrate such as starches and amorphous celluloses were utilized contributing to early weight gain. Then as the substrate becomes less nutritious, the weight gain stagnated and even decreased in some cases as the larvae began to live primarily off of their fat stores.

This is supported by comparing the ‘Dry % Substrate Utilized by Larvae’ in **Tables 5-8 to 5-12** and their respective **Figs. 5-20 to 5-24** larval weight gain, with the exclusion of day 12 for the *Trichoderma koningii* sample for reasons explained below. The weight of the larvae increased as the substrate was utilized, but as the larval digestion period continued, the concentration of cellulose rose and the substrate became less nutritious. Both larval weight gain and substrate utilization were then retarded. Since the dry weight of the larvae was not measured, the actual incorporation of substrate into larval biomass from these results is unknown. Some of the digested substrate was also utilized for larval metabolic and kinesiological processes, and was lost as CO<sub>2</sub> to the atmosphere.

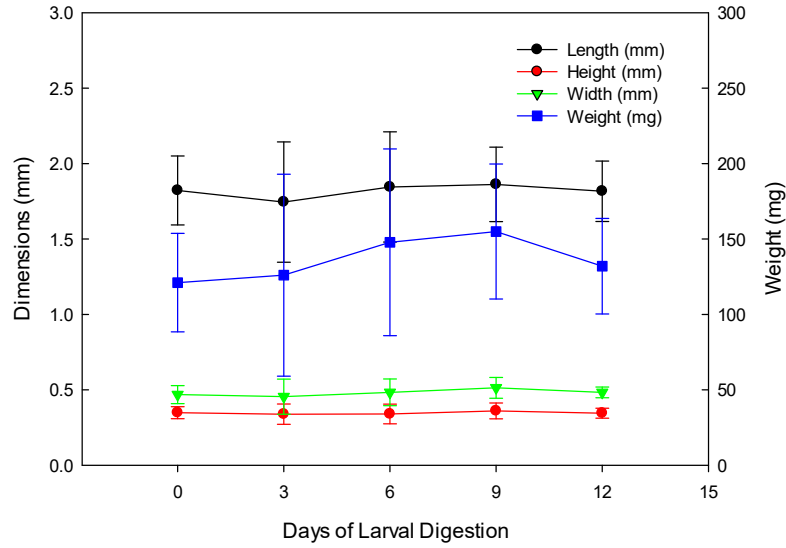


**Fig. 5-20 Larval Parameters *Phanerochaete chrysosporium* SSF+Larvae**

N = 6; mean and std. dev.;

**Table 5-4 System Changes in *Phanerochaete chrysosporium* SSF + Larvae**

Day	0	3	6	9	12
% Substrate Utilized by Larvae (dwb)	0	33	33	39	40
Weight of Larvae Under Unchanging Numbers (mg, ww)	100	125.9	111.1	101.9	110.9

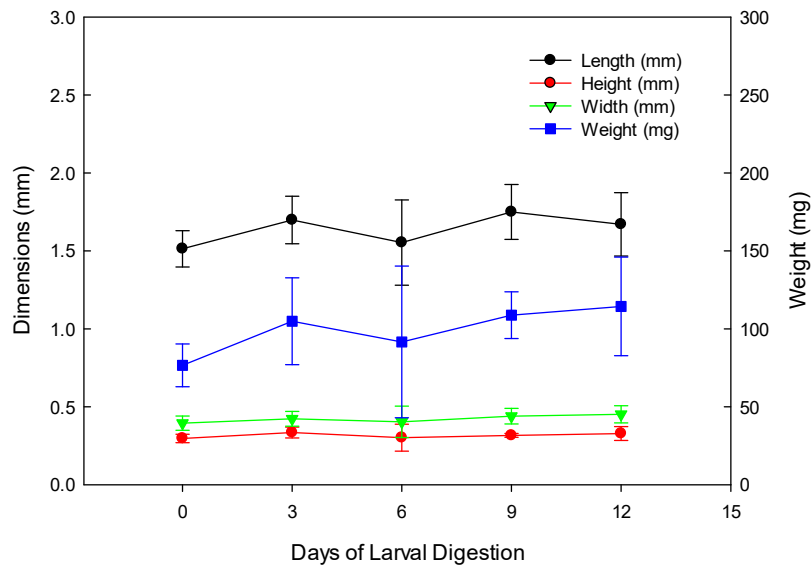


**Fig. 5-21 Larval Parameters *Trichoderma koningii* SSF+Larvae**

N = 6; mean and std. dev.;

**Table 5-5 System Changes in *Trichoderma koningii* BRRL54330 SSF + Larvae**

Day	0	3	6	9	12
% Substrate Utilized by Larvae (dwb)	0	11	39	55	95
Weight of Larvae Under Unchanging Numbers (mg, ww)	100.0	104.1	122.1	128.0	109.0

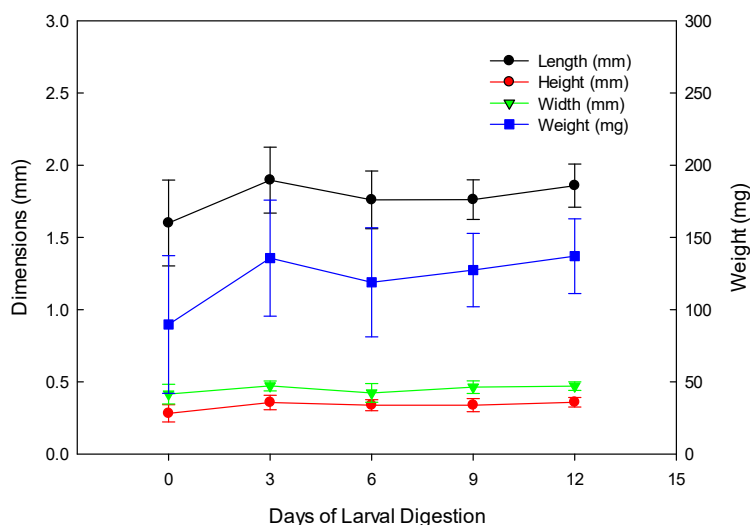


**Fig. 5-22 Larval Parameters *Aspergillus fumigatus* SSF+Larvae**

N = 6; mean and std. dev.;

**Table 5-6 System Changes in *Aspergillus fumigatus* BRRL 163 SSF + Larvae**

Day	0	3	6	9	12
% Substrate Utilized by Larvae (dwb)	0	34	44	59	69
Weight of Larvae Under Unchanging Numbers (mg, ww)	100	137.0	119.7	142.0	149.4



**Fig. 5-23 Larval Parameters *Aspergillus niger* BRRL 322 SSF+Larvae**

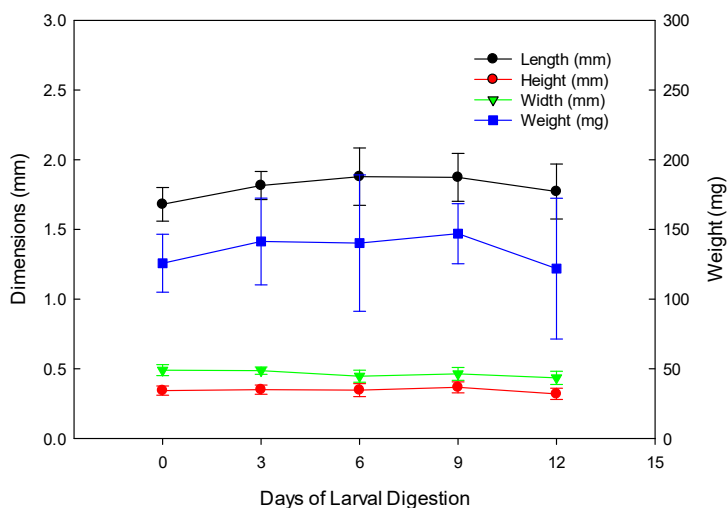
N = 6; mean and std. dev.;

**Table 5-7 System Changes in *Aspergillus niger* BRRL 322 SSF + Larvae**

Day	0	3	6	9	12
% Substrate Utilized by Larvae (dwb)	0	24	41	46	68
Weight of Larvae Under Unchanging Numbers (mg, ww)	100	111.7	104.3	124.7	126.0

The last contention with larval size data is that the larvae were not known to be of a certain generation since they did not all come from the same clutch of eggs, or collection of eggs at a similar time period. The only criteria for being utilized in the digestion studies was that they had not yet begun to pupate. Thus, they are likely in their 4<sup>th</sup> or 5<sup>th</sup> instars. As larvae pupate, they shed their digestive systems, develop a hard chitin exoskeleton (to protect from predation; it also becomes dark brown and blends into soils and typical natural pupation substrates), and become immobile during metamorphosis. There are 5 instars in total in the black soldier fly larvae, between which they molt their exoskeleton, and excrete a new larger one to allow their bodies to

expand in size. Smaller larvae from earlier instars have far greater opportunity for size gains, while 4<sup>th</sup> and 5<sup>th</sup> instars larvae are approaching their maximum body weight, and while they may contribute greatly to substrate reduction/degradation, will not incorporate the substrate into dry larval biomass much at all. The substrate is used to power their higher metabolic needs at that size. Due to using late instar larvae, the total gain in larval biomass was less than what can be expected when utilizing less mature larvae, but mature larvae still an effect on the substrate through their digestion of the substrate.



**Fig. 5-24 Larval Parameters *Lactobacillus plantarum* SSF+Larvae**

N = 6; mean and std. dev.;

**Table 5-8 System Changes in *Lactobacillus plantarum* SSF + Larvae**

Day	0	3	6	9	12
% Substrate Utilized by Larvae (dwb)	0	33	42	44	70
Weight of Larvae Under Unchanging Numbers (mg, ww)	100	112.4	111.5	116.9	96.9

As the time of larval digestion increased, a larger percent of the dry matter of the substrate was utilized by the larvae. The larvae reduced the substrate dry matter by utilizing it as a feed source for their metabolic processes. The total utilization of the substrate is not consistent across samples. *Trichoderma koningii* showed the greatest amount of substrate utilization, followed by *Lactobacillus plantarum*, *Aspergillus niger*, *Aspergillus fumigatus*, and finally by *Phanerochaete chrysosporium*. The total substrate utilization seen on **Table 5-5** on day 12 of the *Trichoderma koningii* sample was 95%. This was much higher than the other samples, and is

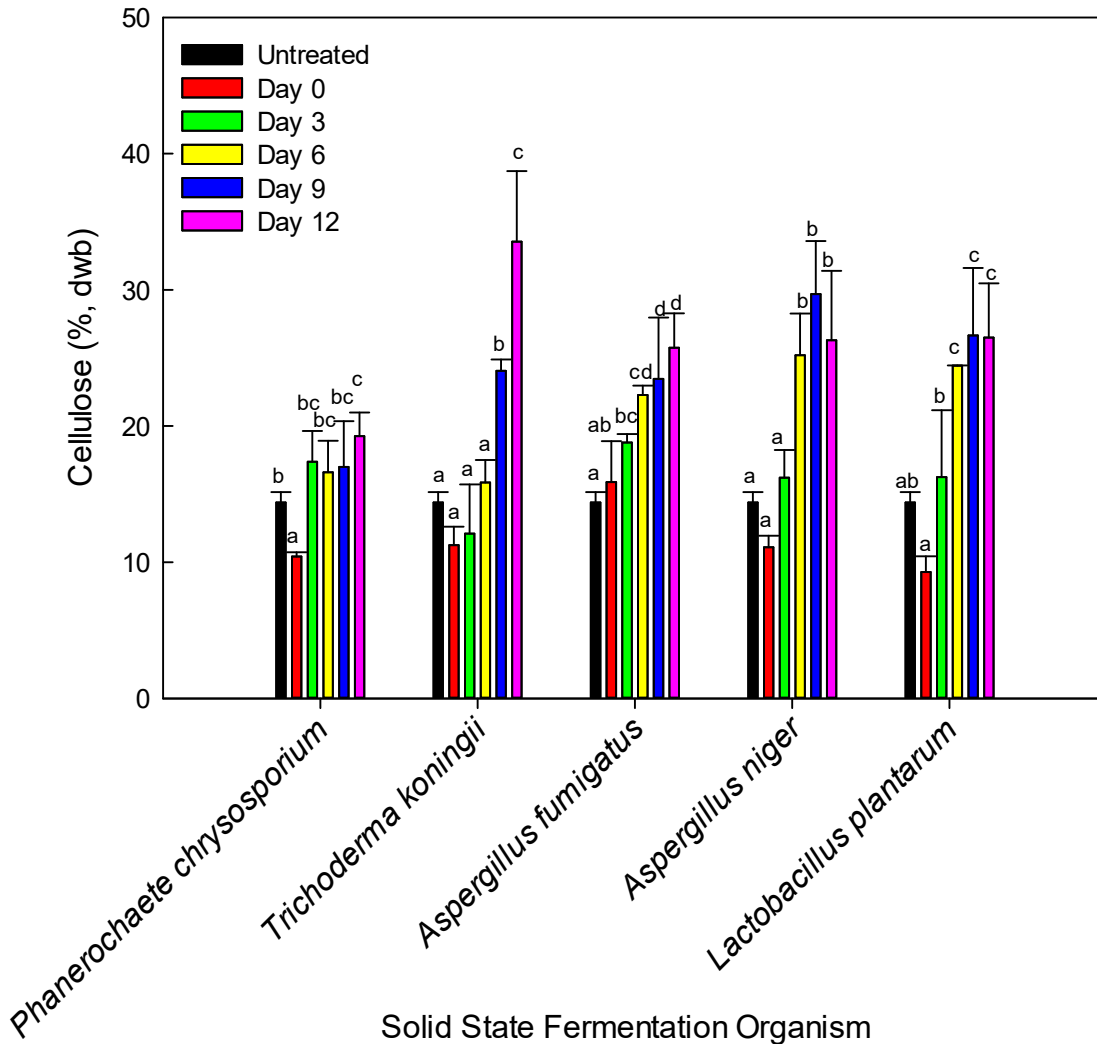


questionable given that the substrate had a cellulose content of between 11.3 to 33.5% (dwb) during the digestion period and that BSFL are inefficient cellulose digesters. This is likely an outlier caused by removing too much substrate during earlier testing days, giving the false impression that the larvae had consumed a lot more than they had. The data is reasonable up to day 9 in this sample. Total substrate utilization by BSFL approached 70% by day 12 of larval digestion for most samples.

The trend of DDGS substrate utilization by the BSFL matched the trend of protease production by SSF organisms from the earlier SSF studies on DDGS here in Study 2 (**Fig. 5-14**). The protease activity in the post-SSF samples of 100% DDGS went in the order from highest to lowest of *Aspergillus niger*, *Trichoderma koningii*, *Aspergillus fumigatus*, and *Phanerochaete chrysosporium*. As DDGS contains a high percentage of protein, these high protease producing organisms provided a superior degradation of the substrate. This opening of the DDGS structural matrix may also have a synergistic effect, allowing the other enzymes greater access to the other constituents in DDGS, notably cellulose. This is supported by the fact that, although the order of cellulase activity in post-SSF homogeneous 100% DDGS was *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Trichoderma koningii*, the cellulose content of these substrates was inverse to this relationship, with the exception of *Aspergillus niger*.

This makes sense as protein is present in a much higher percentage than D-glucose cellulose homopolymers (which the cellulose test measured). Therefore protease activity had a greater impact on the opening of the DDGS matrix than cellulase. It also allowed for greater substrate degradation overall, including that of cellulose. SSF organisms which contribute to the greatest amount of substrate degradation result in the most nutrient available feed for larval feeding. Additionally, fungal biomass which is manufactured during the SSF process is readily digested by larvae, making cleanup from the SSF process less of an issue. BSFL are naturally saprophagous and therefore well suited to competition with natural waste digesting microorganisms.

The cellulose content of the substrates was reduced following SSF, except for *Aspergillus fumigatus* which experienced a slight but statistically insignificant increase in cellulose content as shown on **Fig. 5-25**. The cellulose content was reduced the most for *Aspergillus niger*. When comparing the SSF samples to the control DDGS sample which had identical treatment, but with



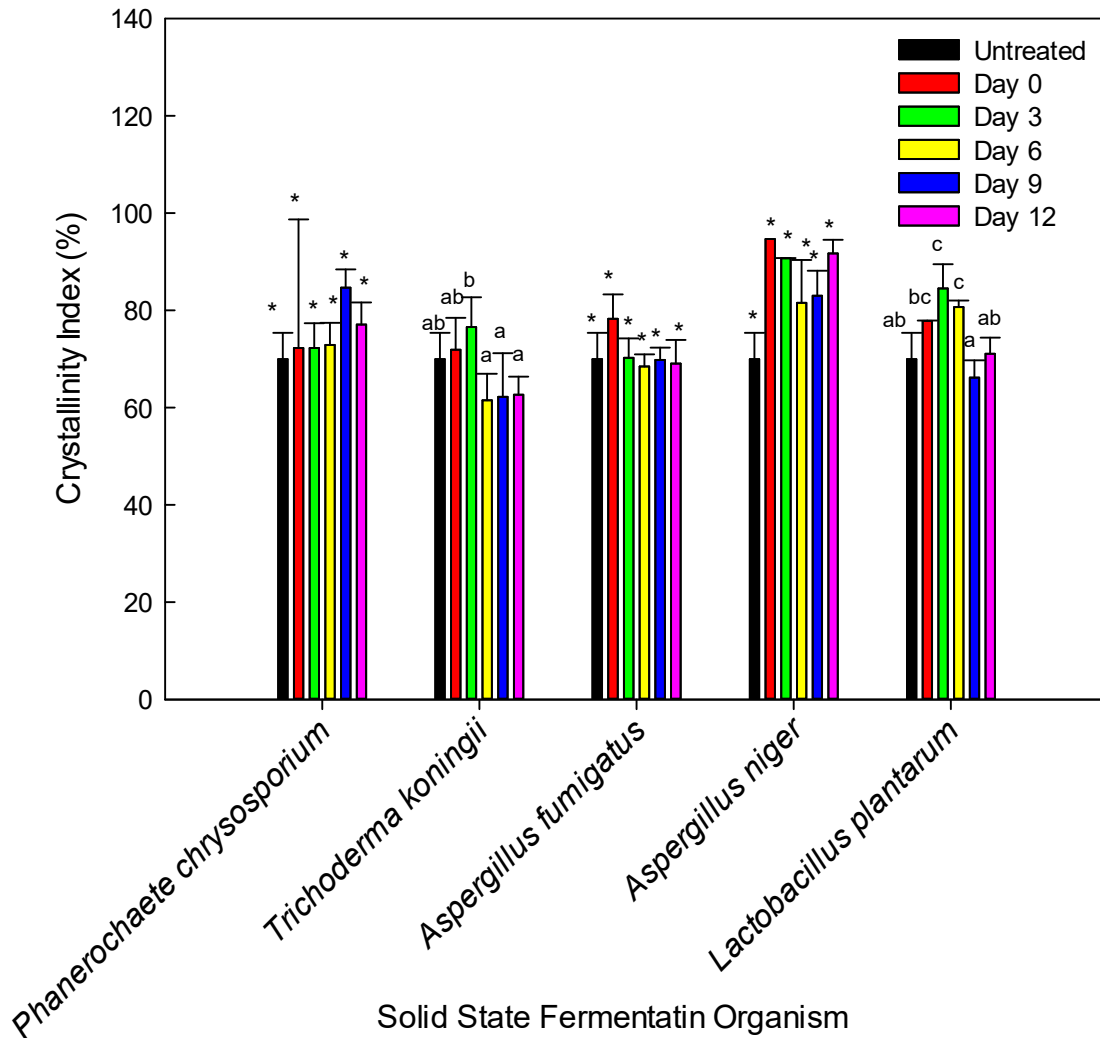
**Fig. 5-25 Cellulose Content of SSF+Larvae during 12 Day Larval Digestion**

Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the solid state fermentation (SSF) period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD within groupings; for *Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus fumigatus*, *Aspergillus niger*, *Lactobacillus plantarum*  $p = 0.003$ ,  $< 0.001$ ,  $= 0.001$ ,  $< 0.001$ ,  $< 0.0011$  respectively and LSD = 3.67, 4.91, 4.38, 5.43, 5.91 respectively;

a sterile SSF period, the SSF samples did not reduce the cellulose content more than the control sample. Control cellulose reduction went from 14% for untreated DDGS to 9% for control DDGS after the sterile SSF period. This is similar to all SSF samples, except for *Aspergillus fumigatus*, and suggests that although the SSF organisms produce cellulase and have a degradative effect on the cellulose fibers, they do not convert a large amount to amorphous cellulose during the SSF period. An analysis in this study on SSF of DDGS (**Fig. 5-12**) showed that *A. niger* and *T. koningii* could reduce the cellulose content of homogeneous ground DDGS to around 5%. The conditions of the SSF for use with BSFL were slightly different, mainly having a greater moisture content, indicating an area for improvement and optimization with regards to cellulose degradation during SSF. The cellulose content reduction here can be attributed primarily to swelling of the cellulose fibers during the SSF period as discussed in Section 5.3.1. This contributed to a general 5% reduction in cellulose content.

A major finding of SSF combined with BSFL digestion experiments is that the cellulose content of DDGS was concentrated by larval digestion. The relative cellulose content of the substrate increased significantly during larval digestion for all samples evaluated as shown on **Fig. 5-25**. From looking at the system change data on **Tables 5-8** to **5-12**, it was seen that as larval time increases, reduction in the dry matter is seen. Looking at the cellulose content data, it can be seen that the undigested substrate is highly composed of cellulose. This is supported by anecdotal evidence which states that BSFL are poor utilizers of cellulosic materials.

The increase in cellulose content was not high enough to say that the cellulose was not utilized at all; rather it was resistant to digestion and became more concentrated over time as other substrate constituents were metabolized by the larvae. This increase in cellulose content was the least among SSF feeds for *Phanerochaete chrysosporium*, which displayed the greatest cellulase activity. This makes sense as cellulase would make the cellulose fraction more susceptible to the BSFL's digestive system. Cellulase decreases the crystallinity of cellulose, and amorphous cellulose is more susceptible to degradation and removal by the BSFL's digestive system.



**Fig. 5-26 Crystallinity Index of SSF+Larvae during 12 Day Larval Digestion**

Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the solid state fermentation (SSF) period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD within groupings; for *Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus fumigatus*, *Aspergillus niger*, *Lactobacillus plantarum*  $p > 0.05$ , = 0.054, > 0.05, > 0.05, = 0.001 respectively and LSD = N/A, 11.09, N/A, N/A, 8.15 respectively;

The SSF organisms which have the greatest protease activity, *Aspergillus Niger* and *Trichoderma koningii*, also showed the fastest increase in cellulose content during the larval digestion period. Since DDGS is highly comprised of proteins, these constituents will be greatly hydrolyzed and susceptible to BSFL digestion, facilitating their expedient removal. DDGS

protein concentrations are nearly three times the concentration of cellulose which gives protease activity more weight in the overall DDGS degradation. The cellulose content for the *Lactobacillus plantarum* treated sample showed a comparable increase in cellulose content over the larval digestion period. Given that it displayed no cellulase activity, but did display high protease activity, as seen on **Table 4-2** culture #5, this gives more weight to the theory that high protease activity plays a large role in facilitating the concentration of cellulose through the expedient digestion of DDGS proteins. This increase in cellulose content of the substrates is strongly correlated and partially causal of the increase in sugar liberation by acidic and enzymatic hydrolysis.

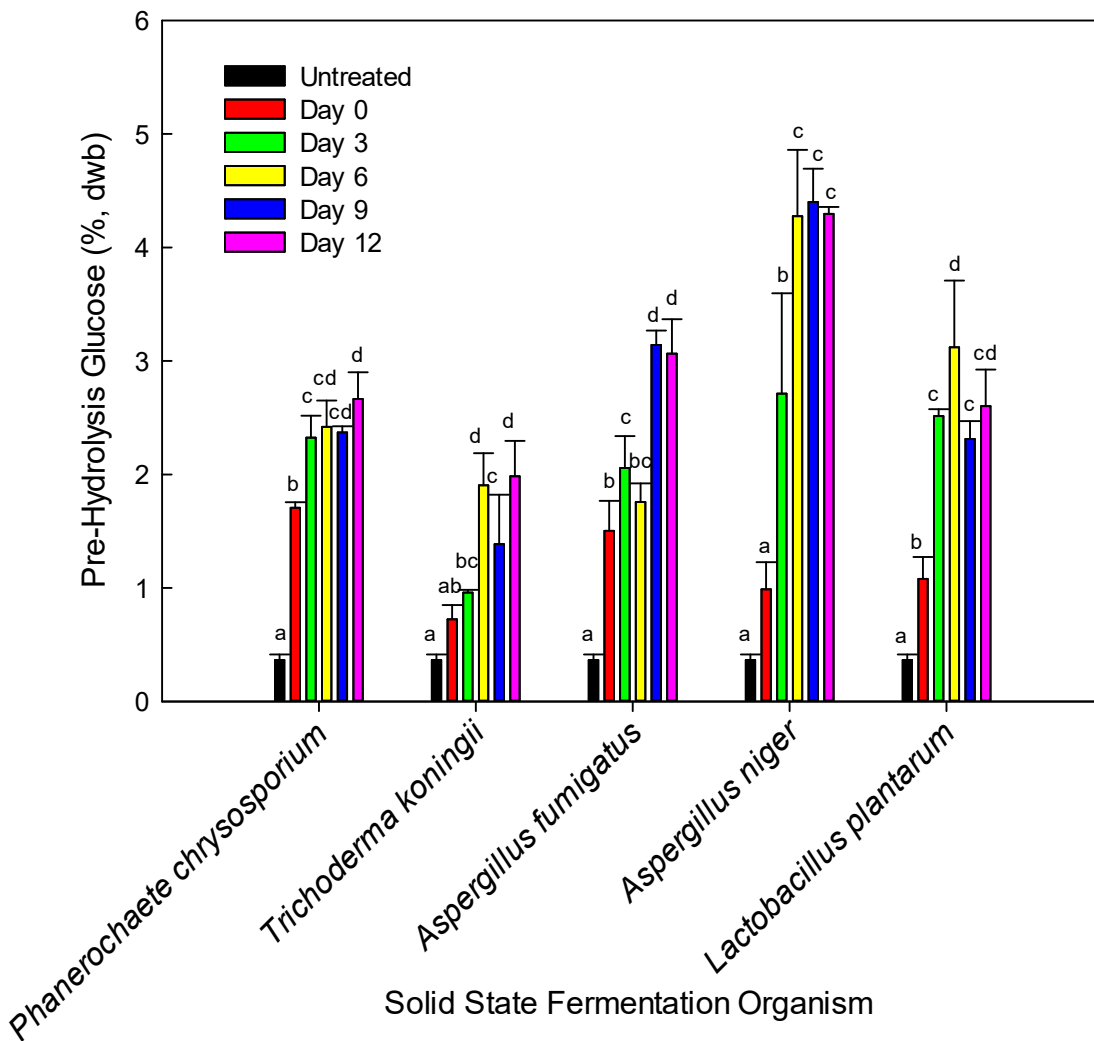
As shown on **Fig. 5-26** the cellulose crystallinity index was increased by SSF, and subsequently decreased by larval digestion. The change was not significant for any SSF organism following either SSF or 12 day larval digestion as compared to the untreated sample. *Trichoderma koningii* and *Lactobacillus plantarum* both showed a significant increase by day 6 of larval digestion. This again shows that SSF does not increase the ratio of amorphous to crystalline regions of cellulose. This may be due to a lack of de-crystallization, or more likely to a removal of amorphous regions due to fungal enzyme activities. Larval digestion showed a significant decrease in cellulose crystallinity from days 3 to 12 for samples *Trichoderma koningii* and *Lactobacillus plantarum* as well as a general decrease in *Aspergillus fumigatus* and *Aspergillus niger* up to day 9 although these were not statistically significant.

The concentration of free glucose in the substrates represents a substantial amount of the carbohydrates in DDGS as seen on **Fig. 5-27**. SSF increased this amount significantly for all samples except *Trichoderma koningii*, and *Aspergillus niger*. The amount of free glucose in the substrate was also increased over the larval digestion period. This may be due to continuing action of the extracellular enzymes excreted during SSF, as well as larval digestive processes. This increase was similar to the control DDGS + Larvae sample over the larval digestion period (**Fig. 5-5**). However, SSF produced nearly double the amount of free glucose than the control except in the case of *Trichoderma koningii* which was nearly identical to the control. Larval digestion produced similar amounts of free glucose compared to non SSF DDGS. *Aspergillus niger* produced much more free glucose, and produced it earlier in the larval digestion period compared to all other samples. This organism produces large amounts of  $\beta$ -glucosidase, an enzyme that cleaves bio-cellulose into glucose. This may explain the higher free glucose

production in that sample. The present research supports previous research on the effect of BSFL digestion of dairy manure<sup>190</sup> as the crystallinity index was shown to decrease over the larval digestion period. This was due to an opening of the structure due to enzymes present from SSF and from larval mastication which physically shears cellulose fibers. This leads to an increase in amorphous cellulose regions which are released during larval digestion and acidic hydrolysis as glucose. This is shown in the large increase in pre-hydrolysis glucose, as well as increasing acidic glucose liberation values which are greater than either the non fermented DDGS, or SSF periods alone (i.e., Day 0).

The amount of glucose which can be liberated from DDGS via dilute acid hydrolysis is shown on **Fig. 5-28**. It did not change significantly for the sample processed using *Aspergillus fumigatus* during SSF or larval digestion; although a slight increase was observed. *Phanerochaete chrysosporium* was the only fungal sample which resulted in a greater amount of glucose being liberated following SSF which is noted by comparing values for untreated DDGS and day 0 samples. *Phanerochaete chrysosporium*, *Trichoderma koningii*, and *Aspergillus niger* larval digestion significantly increased the acid hydrolysis glucose yield as the larval digestion time increased. This may be due in large part to the cellulose concentrating effect of larval digestion, rather than a change to the properties of the substrate. The values for acidic sugar liberation are rather low compared to the increase in cellulose content so there are other factors at play here as well. In general, SSF was not found to generate large amounts of amorphous cellulose, which is the primary source of dilute acid hydrolysis glucose.

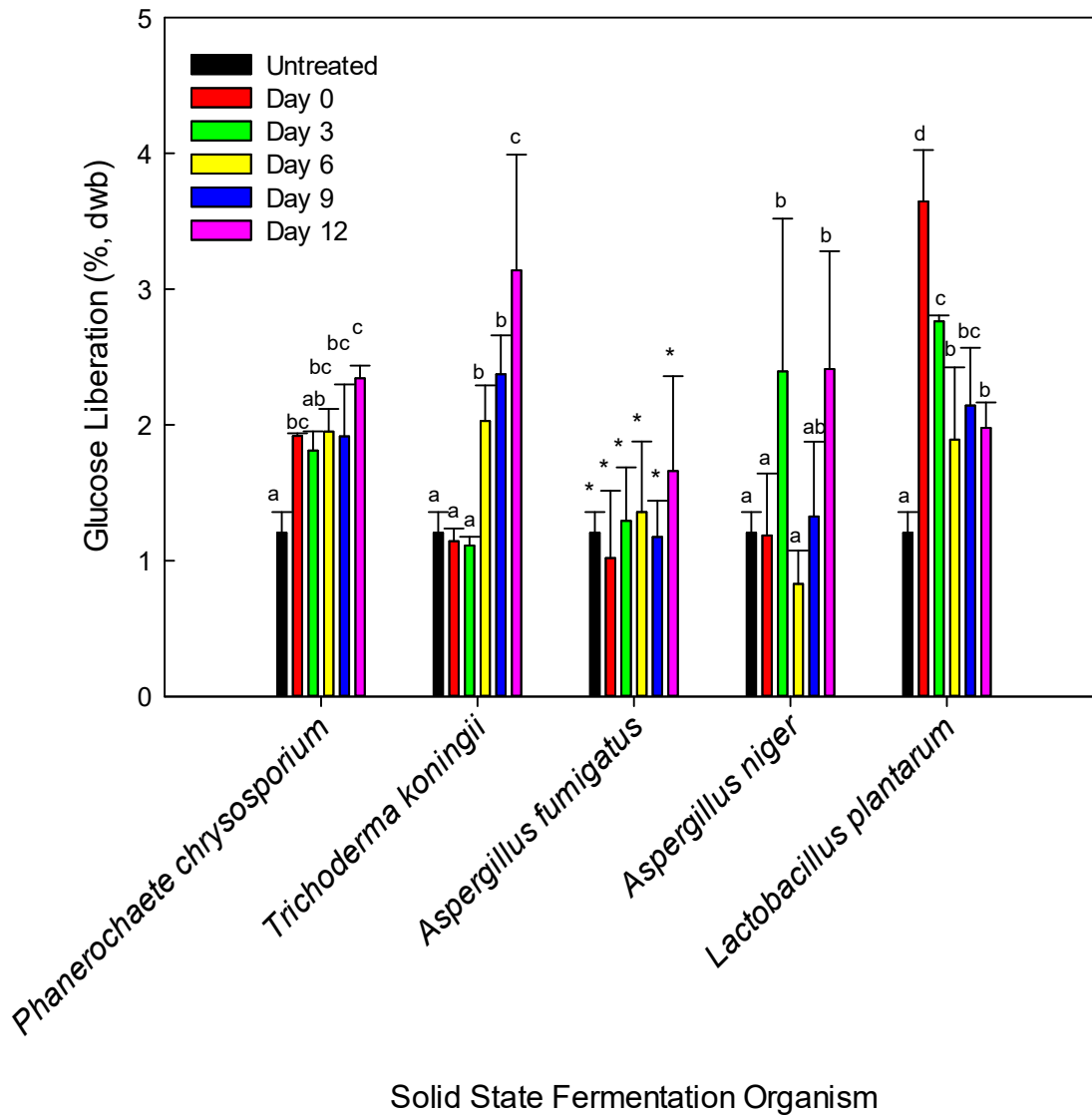
A low free-glucose and high acidic hydrolysis following the control SSF period indicated that DDGS contains a high amorphous cellulose and/or starch content as discussed in 5.3.2. Fungal SSF (**Figs. 5-27** day 0 and **5-28** day 0) produced higher free glucose and lower acidic hydrolysis glucose yields than the control DDGS treatment. This indicates that the amorphous cellulose was cleaved from DDGS and was, along with starches, hydrolyzed to glucose during SSF. These sugars were partially used by the SSF organisms during the SSF period, but since the control DDGS contained no fermentation organism, day 0 acid hydrolysis yields were very high for the control sample.



**Fig. 5-27 Free-Glucose in Spent Feed from SSF+Larvae during 12 Day Larval Digestion**

Glucose % = [mg glucose/(mg dry wt. of substrate \* 0.507)] \* 100%; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Pre-hydrolysis or free-glucose is a measure of the soluble glucose concentration of a substrate. In DDGS, it is produced during larval digestion and solid state fermentation (SSF) via enzymatic actions of the Black Soldier Fly larvae (BSFL) and SSF organisms; and via mechanical hydrolysis by the BSFL of bound sugars, starches, and amorphous cellulose. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the SSF period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD within groupings; for *Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus fumigatus*, *Aspergillus niger*, *Lactobacillus plantarum*  $p < 0.001$ ,  $< 0.001$ ,  $< 0.001$ ,  $< 0.001$ ,  $< 0.001$  respectively and LSD = 0.29, 0.45, 0.39, 0.82, 0.52 respectively;

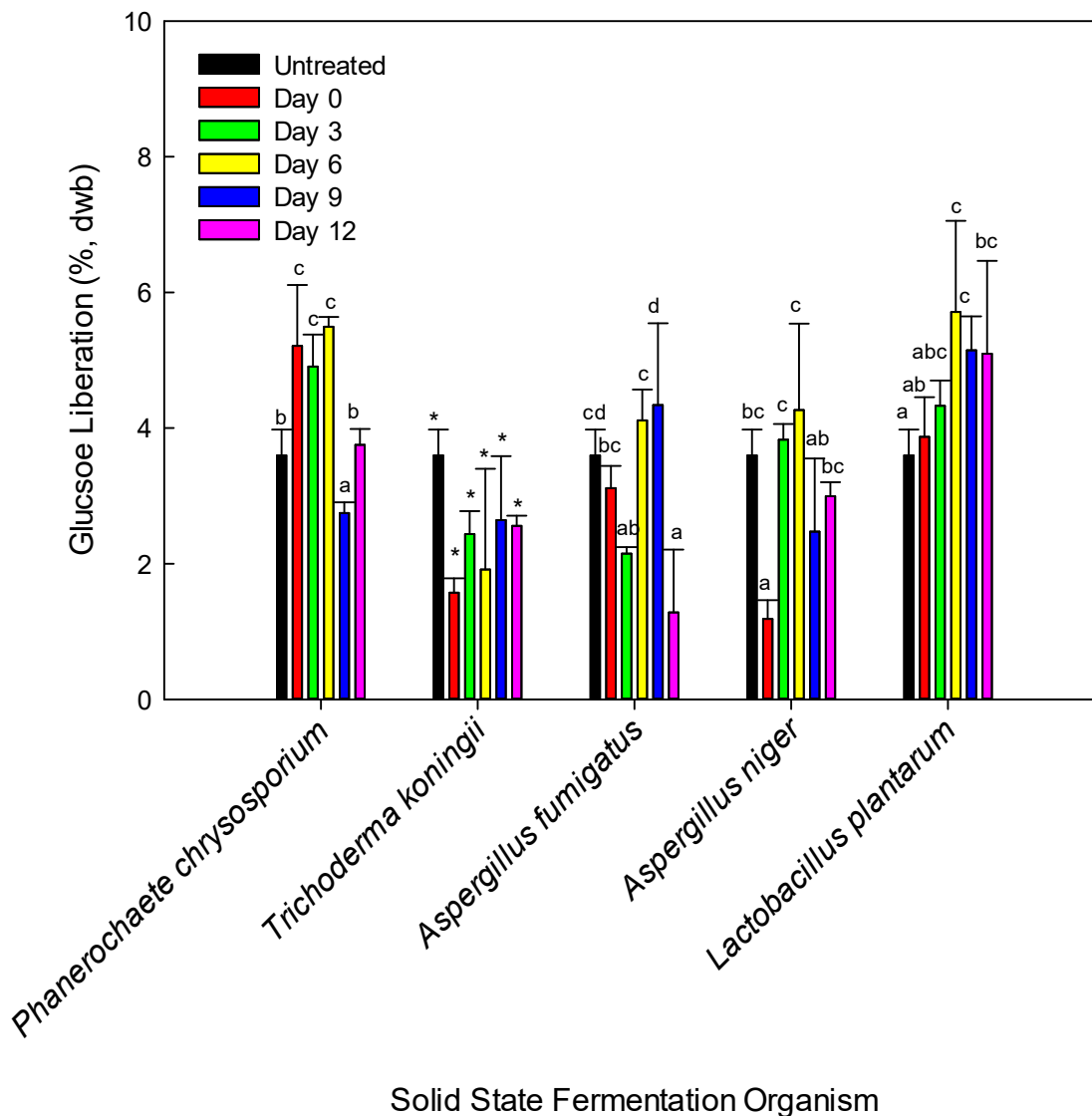
Of particular interest is the *Lactobacillus plantarum* acid hydrolysis data. A large and significant increase in sugar liberation was seen following the SSF period which followed a similar trend to the control SSF sample on day 0. A large initial glucose yield was followed by a decrease and then fluctuation around 2% for days six through twelve. This pattern of sugar



**Fig. 5-28 Sugar Liberation by Acid Hydrolysis of Spent Feed from SSF+Larvae during 12 Day Larval Digestion**

Glucose % = [mg glucose/(mg dry wt. of substrate \* 0.507)] \* 100%; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Glucose liberated from acid hydrolysis is a measure of the structurally weak glucose concentration of a substrate such as starches and amorphous cellulose. SSF and BSFL quickly hydrolyze starches. Longer larval digestion times therefore show primarily amorphous cellulose glucose. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the solid state fermentation (SSF) period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD within groupings; for *Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus fumigatus*, *Aspergillus niger*, *Lactobacillus plantarum*  $p = 0.019$ ,  $< 0.001$ ,  $> 0.05$ ,  $= 0.049$ ,  $< 0.001$  respectively and LSD = 0.63, 0.73, N/A, 1.17, 0.61 respectively;





**Fig. 5-29 Sugar Liberation by Enzymatic Hydrolysis of Spent Feed from SSF+Larvae during 12 Day Larval Digestion**

Glucose % =  $\text{mg glucose} / (\text{mg dry wt. of substrate} * 0.507) * 100\%$ ; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Enzymatic hydrolysis is preceded by dilute acid hydrolysis which removes structurally weak glucose polymers and free-glucose. Glucose liberation via enzymatic hydrolysis (cellulase) is therefore a measure of the amount of sugars which can be recovered from crystalline cellulose particles under the current system. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the solid state fermentation (SSF) period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD within groupings; for *Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus fumigatus*, *Aspergillus niger*, *Lactobacillus plantarum*  $p < 0.001$ , = 0.086, = 0.001, = 0.003, = 0.046 respectively and LSD = 0.82, N/A, 1.21, 1.29, 1.46 respectively;

liberation is speculated to occur for the same reason as the control sample (**Fig. 5-6**). A lack of cellulase to remove the amorphous cellulose combined with a starch response produced large glucose yields following SSF. Then, larval digestion removed the amorphous cellulose, and hydrolyzed starch contributing to high pre-hydrolysis glucose concentrations, but lower acidic hydrolysis yields than day 0. The *Lactobacillus plantarum* treated sample released nearly the same amount of sugar as the control sample immediately following SSF, but released more sugars at every day of larval digestion, indicating that SSF using this organism had a beneficial effect on glucose yields. By comparing the glucose yields from acidic hydrolysis to the crystallinity index of the cellulose, on **Fig. 5-26** we can see that the CI and glucose yields from acid hydrolysis have an inverse relationship for a given SSF organism. An increase in CI means that there is less amorphous cellulose present compared to crystalline cellulose. Amorphous cellulose is cleaved during dilute acid hydrolysis and therefore a higher CI results in lower glucose yields from acidic hydrolysis. Additionally, glucose yields from dilute acid hydrolysis were highly correlated to the amount of cellulose in the sample for fungal SSF treated DDGS as was expected. This relationship was more complex for the *L. plantarum* treated DDGS. Because acidic hydrolysis glucose yields did not change for the control DDGS during the larval digestion phase, the fourth hypothesis of this research, that BSFL can increase acidic sugar yields, was disproven. BSFL digestion did improve total sugar yields, but amorphous cellulose regions were presumably removed during BSFL digestion, leading to higher pre-hydrolysis glucose yields in digested DDGS.

Because the amorphous cellulose was primarily removed during acidic hydrolysis, the enzymatic hydrolysis results represent changes to the porosity in highly crystalline cellulose regions and cellulose particle size. As shown on **Fig. 5-29**, enzymatic hydrolysis of the substrates showed no significant change for the *Trichoderma koningii* sample. The results were also inconsistent across the fungal SSF strains with all other samples seeing either no significant change from the untreated DDGS to day 12, or a significant decrease. *Lactobacillus plantarum* treated DDGS showed an increase in enzymatic hydrolysis glucose yields over the larval digestion period. *Phanerochaete chrysosporium* produced high glucose yields following SSF and during initial larval digestion.

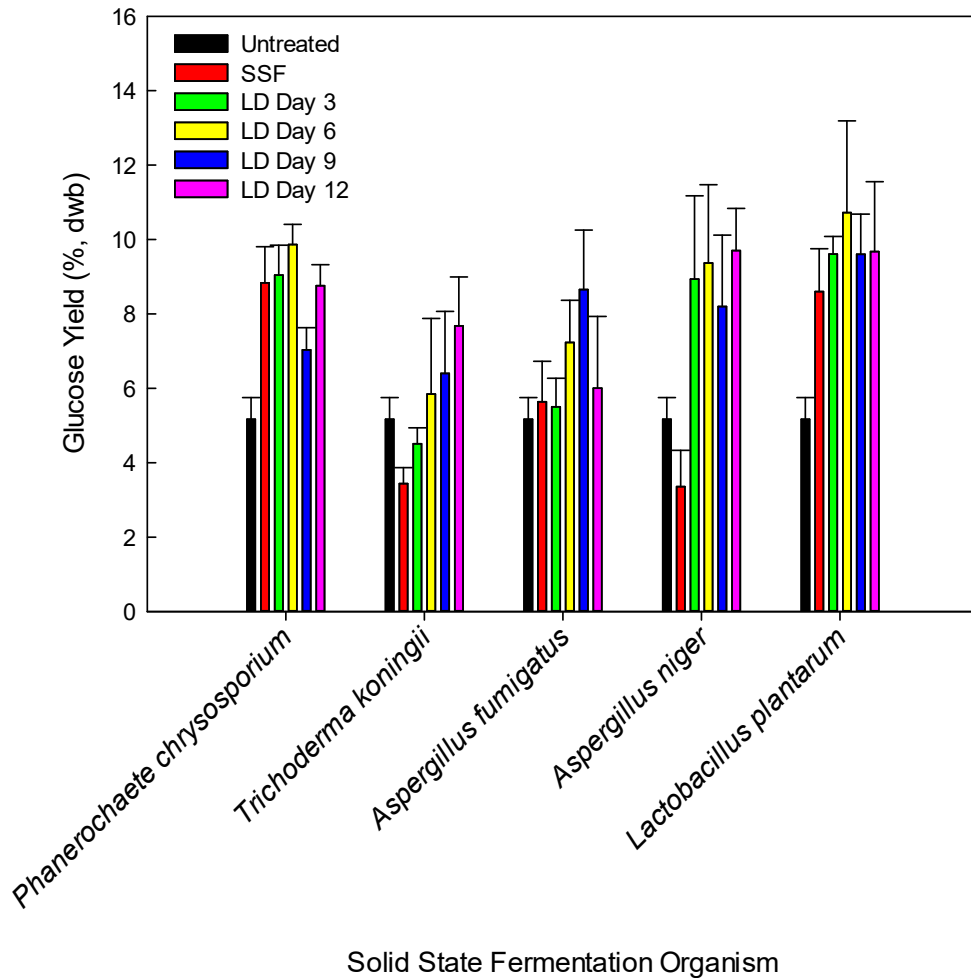
The use of SSF greatly increased the enzymatic glucose yields as compared to unfermented DDGS. All SSF treated samples showed higher glucose liberation as compared to

the control sample as discussed in section 5.3.2., indicating that SSF degraded the cellulose therein making them more susceptible to enzymatic liberation. This proved the third hypothesis of this research that SSF would weaken DDGS cellulose structure and facilitate greater sugar yields via enzymatic hydrolysis. Micronization of cellulose fibers from BSFL digestion also served to increase the surface area of the cellulose fibers and thus their susceptibility towards enzymatic hydrolysis

**Fig. 5-30** shows the complete picture for glucose yields from SSF and larval treated DDGS. Compared to untreated DDGS, *Trichoderma koningii*, and *Aspergillus niger* SSF had decreasing effect on the glucose yields seen by comparing untreated to day 0 glucose yields. *Aspergillus fumigatus* SSF showed a slight increase in glucose yields, while *Phanerochaete chrysosporium* and *Lactobacillus plantarum* SSF greatly increased day 0 glucose yields from untreated DDGS. Larval digestion further increased the total glucose yields from the SSF period, and especially compared to untreated DDGS. The majority of the increase in these samples came from increases to the pre-hydrolysis glucose yields. *Phanerochaete chrysosporium* and *Lactobacillus plantarum* had good hydrolysis yields. *Phanerochaete chrysosporium* SSF produced the lowest cellulose content substrate, indicating that it was slightly superior in degradation of cellulose. Total glucose yields of the control sample were shown at 4.5 - 6.5% earlier in this study (**Fig. 5-8**). SSF along with larval digestion resulted in total glucose yields of 3.5-11%, and were always higher than the control by day 6 of larval digestion for every sample. Most samples showed immediate glucose yield increases over control following SSF and 3 days of larval digestion. Therefore, while the use of BSFL was shown to increase the glucose yields from DDGS over a 12 day larval digestion, the use of SSF helped to increase this yield in every case.

Overall, combination of SSF and larval digestion led to a large increase in the total glucose yields from SSF as compared to the control sample, and especially compared to untreated DDGS. The degradation of DDGS by SSF organisms also allows for greater substrate incorporation of DDGS into larval biomass. This experiment also elucidated the need for sterilization of SSF organisms prior to BSFL digestion, as well as the importance of air flow in BSFL systems.

**Table 5-9** shows the result of the bioconversion study in which very young larvae were used to digest SSF DDGS for the purpose of assessing the impact of SSF on larval growth. The amount of substrate utilized by the larvae varied between samples as seen previously, but was much lower than the 70% shown before. This indicates that young larvae require much less nutrients, and take a longer time to utilize the substrate provided as compared to later instar



**Fig. 5-30 Combined Sugar Yield of Spent Feed of SSF+Larvae during 12 Day Larval Digestion**

Glucose % =  $\text{mg glucose} / (\text{mg dry wt. of substrate} * 0.507) * 100\%$ ; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Combined sugar liberation is a combination of the free-glucose, and yields from both acidic and enzymatic hydrolysis of glucose. Error bars are added std. dev. from each analysis. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the solid state fermentation (SSF) period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation.

larvae. The percent utilized was highest for the control sample, and the *Aspergillus fumigatus*, the latter of which was due to a high survivorship among larvae. The control sample also showed the best substrate utilization when using mature larvae. Those that survived in the *Phanerochaete chrysosporium* sample were small compared to those in other samples. Therefore, while they had grown considerably, the total weight of larvae from start to finish decreased resulting in a negative substrate incorporation % for that sample.

Aside from that, SSF actually increased the incorporation of substrate into larval biomass as compared to the control sample. This is not due to larval competition either since the amount of food provided for each larvae was well in excess of what they could eat, and the area of the container was sufficiently large to allow for access to feed. The survivorship of larvae was low, indicating that young larvae are not equipped to digest SSF feed directly. The microorganisms overwhelmed the larvae's immune system killing them before they had a chance to mature. Among those that did mature, SSF DDGS provided a good feed. This indicates the need for sterilization of SSF feed prior to larval feeding when using young larvae.

**Table 5-9 Bioconversions of SSF DDGS into BSFL**

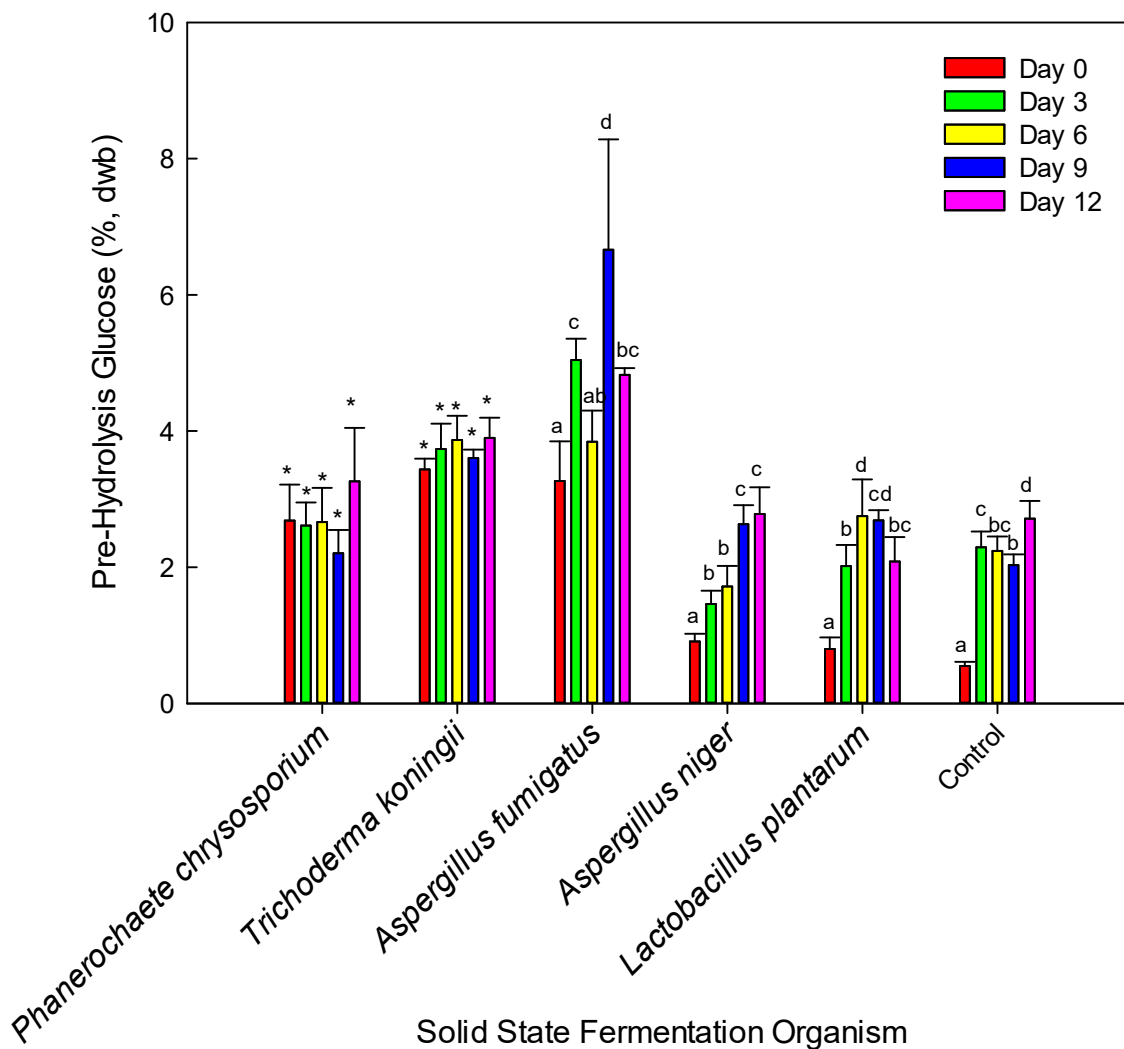
SSF Organism	Dry % Substrate Utilized by Larvae	Final Dry Weight of Larvae Under Unchanging Numbers (mg)	Substrate Incorporation % at 100% Survival (Increase in larval biomass (g, dwb)/starting feed (g, dwb))	Survivorship (%)
<i>Phanerochaete chrysosporium</i> (Mean ± Std. Dev.)	9.3 ± 1.1	3.2 ± 0.8	-14.5 ± 7.0	15.1 ± 15.6
<i>Trichoderma koningii</i> (Mean ± Std. Dev.)	28.5 ± 29.4	14.8 ± 3.2	79.1 ± 26.1	5.3 ± 3.7
<i>Aspergillus fumigatus</i> (Mean ± Std. Dev.)	45.0 ± 8.9	12.1 ± 2.6	55.2 ± 18.3	41.8 ± 13.5
<i>Aspergillus niger</i> (Mean ± Std. Dev.)	26.0 ± 7.4	15.4 ± 2.8	80.4 ± 17.3	7.8 ± 3.9
<i>Lactobacillus plantarum</i> (Mean ± Std. Dev.)	27.5 ± 12.6	14.8 ± 4.4	75.5 ± 30.2	9.5 ± 1.9
Control (Mean ± Std. Dev.)	47.9 ± 3.9	12.4 ± 1.1	43.7 ± 9.0	14.2 ± 3.1

### 5.3.4 Optimization of Sugar Liberation from SSF and Larval Digested DDGS

Glucose liberation experiments were optimized for several process parameters, and then SSF and larval digestion samples were re-tested. The following shows the result of these optimized sugar liberation experiments. The optimization of the conditions for acidic hydrolysis of DDGS further increased the yields of glucose from DDGS carbohydrates via a harsher acidic hydrolysis treatment. Higher temperatures were shown to increase the amount of glucose

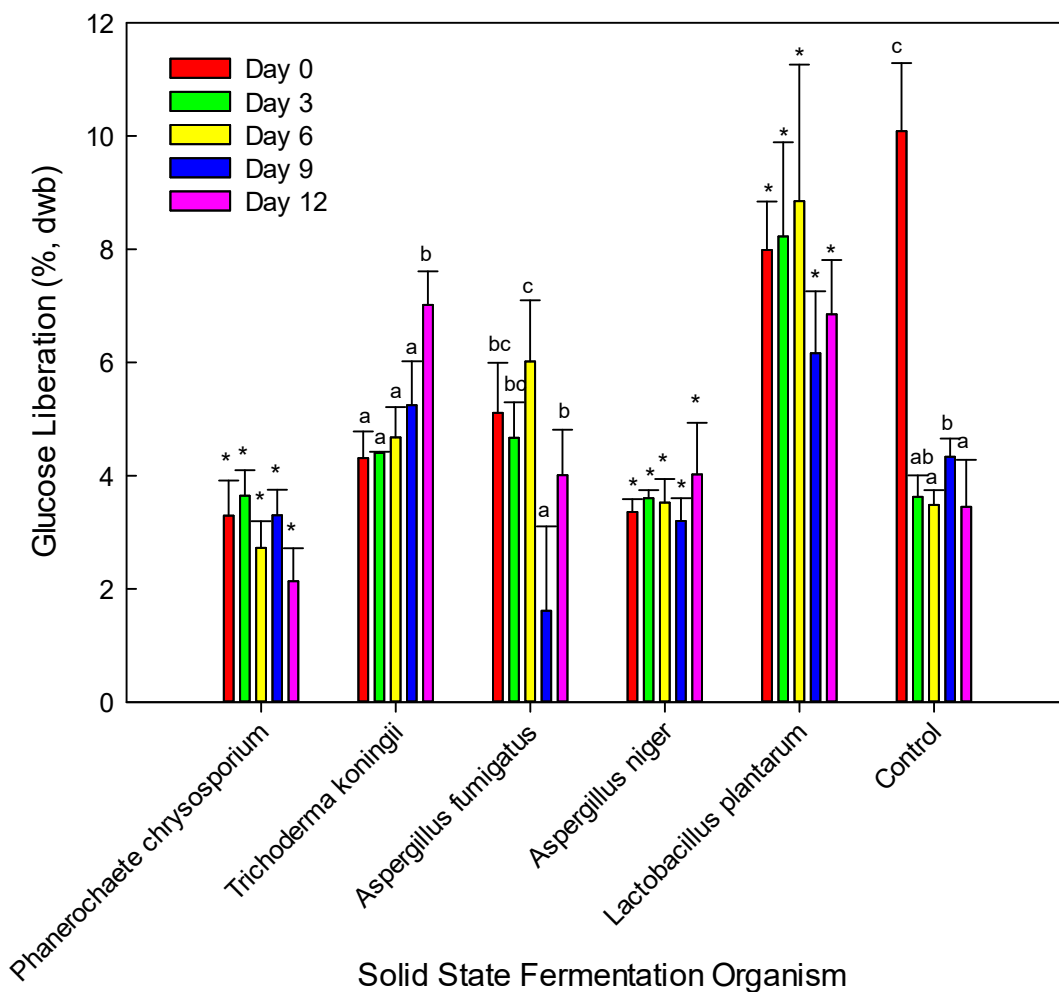
released during hydrolysis. Using screw-cap tubes created a sealed area during hydrolysis. This increased the pressure within the tube as the temperature increased due to expansion of the gasses in the headspace, and as the vapor pressure in the tube rose. This higher pressure led to a higher boiling temperature of the dilute acid solution which further helped to drive hydrolysis of cellulose. Under non-optimized acidic hydrolysis conditions, a closed vessel was used to prevent evaporation and concentration of the analytes during hydrolysis. These vessels were not screw-cap and were therefore not airtight. So while the pressure in those were greater than atmospheric, leading to both a higher boiling point and higher solution temperature than in an open vessel, this effect did not likely occur to the same extent under non-optimized conditions. The substrate concentration was changed from 60 mg/mL to 100 mg/mL, as this substrate loading was shown to be the optimal concentration for glucose production. The pre-hydrolysis sugars from the acid hydrolysis optimized run of the sugar liberation experiments, shown on **Fig. 5-31** again indicated that larval digestion provided an increase in the free glucose.

The amount of glucose released from acidic hydrolysis of the control DDGS, which had undergone a sterile SSF treatment, was dramatically increased by optimizing the hydrolysis conditions (**Fig. 5-32**). Day 0 control DDGS acidic hydrolysis glucose yields went from 4 to 10% after optimization. Glucose yields for other days in the control sample went from around 1% under non-optimized acidic hydrolysis to around 4% under the optimized conditions. For all SSF treated samples, increases to acidic hydrolysis glucose yields were between 2-3 times greater under optimized conditions. The *Phanerochaete chrysosporium*, *Trichoderma koningii*, and *Aspergillus fumigatus* samples used for the optimized glucose liberation experiments were prepared fresh, while the other three samples were analyzed using the same substrates for non-optimized and optimized. The trend in glucose yields from day to day during larval digestion for a given SSF sample was nearly identical from the non-optimized to the optimized acid hydrolysis results for all samples, but the differences between days were more exaggerated in the optimized version. This indicates a good level of reproducibility for SSF and larval digestion of DDGS. The major difference is that the day 0/post-SSF *Lactobacillus plantarum* glucose yield was not significantly greater than all other days for that sample as it was for the control sample. *Lactobacillus plantarum* showed the greatest overall glucose yield. The control sample showed a strong day 0 glucose response, due most likely to having intact starch polymers and amorphous cellulose.



**Fig. 5-31 Optimized Free-Glucose in Spent Feed from SSF+Larvae during 12 Day Larval Digestion**

Glucose % = [mg glucose/(mg dry wt. of substrate \* 0.507)] \* 100%; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Pre-hydrolysis or free-glucose is a measure of the soluble glucose concentration of a substrate. In DDGS, it is produced during larval digestion and solid state fermentation (SSF) via enzymatic actions of the Black Soldier Fly larvae (BSFL) and SSF organisms; and via mechanical hydrolysis by the BSFL of bound sugars, starches, and amorphous cellulose. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the SSF period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD within groupings; for *Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus fumigatus*, *Aspergillus niger*, *Lactobacillus plantarum*  $p > 0.05$ ,  $> 0.05$ ,  $= 0.004$ ,  $< 0.001$ ,  $< 0.001$ ,  $< 0.001$  respectively and LSD = N/A, N/A, 1.47, 0.50, 0.61, 0.24 respectively;



**Fig. 5-32 Optimized Sugar Liberation by Acid Hydrolysis of Spent Feed from SSF+Larvae during 12 Day Larval Digestion**

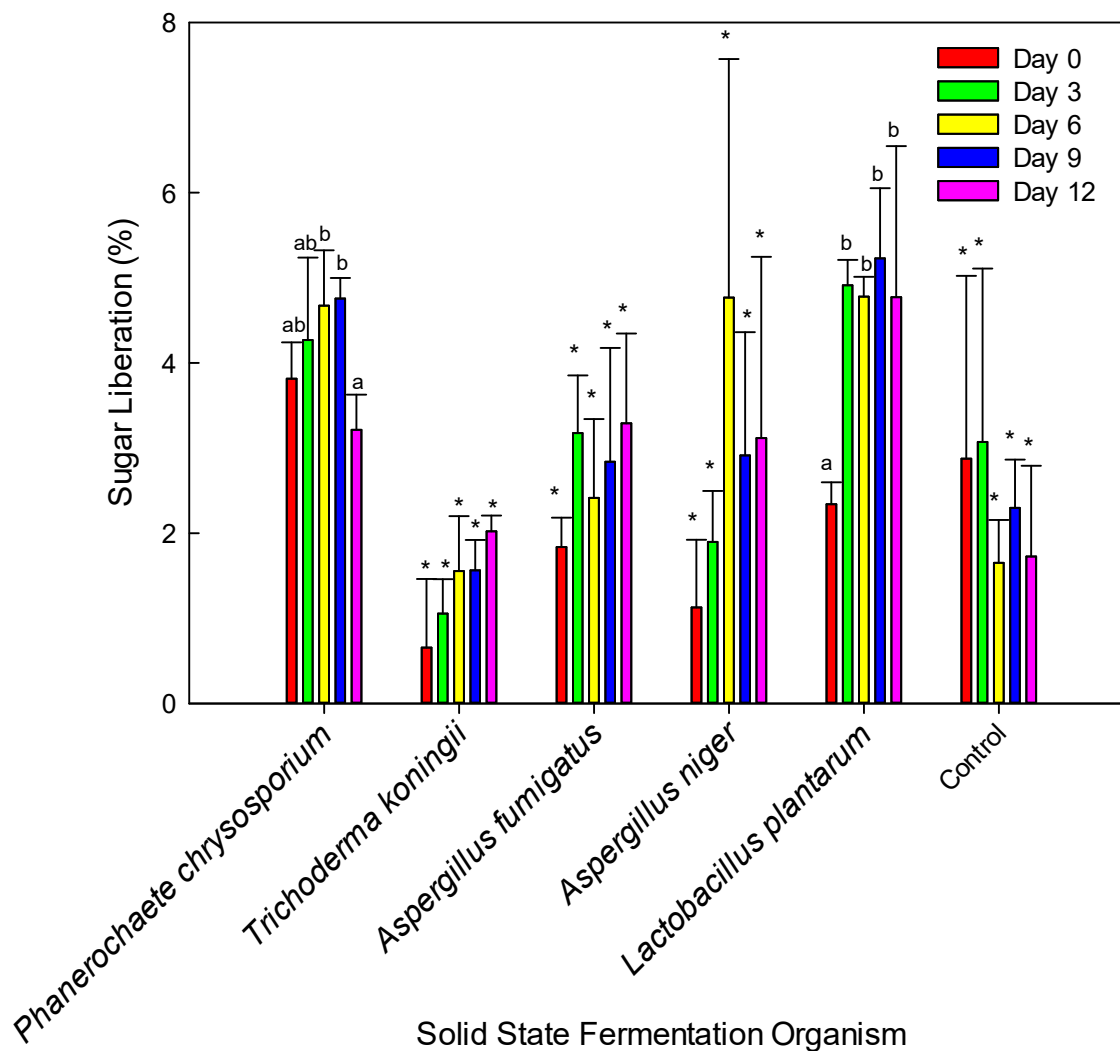
Glucose % = [mg glucose/(mg dry wt. of substrate \* 0.507)] \* 100%; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Glucose liberated from acid hydrolysis is a measure of the structurally weak glucose concentration of a substrate such as starches and amorphous cellulose. SSF and BSFL quickly hydrolyze starches. Longer larval digestion times therefore show primarily amorphous cellulose glucose. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the solid state fermentation (SSF) period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD within groupings; for *Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus fumigatus*, *Aspergillus niger*, *Lactobacillus plantarum*  $p > 0.05$ , = 0.005, = 0.008, > 0.05, > 0.05, < 0.001 respectively and LSD = N/A, 1.23, 1.76, N/A, N/A, 0.83 respectively;



Glucose yields from enzymatic hydrolysis following optimized dilute acid hydrolysis, shown on **Fig. 5-33**, were nearly identical to those following non-optimized dilute acid hydrolysis in both the trend and the amount. The main exception is that control enzyme hydrolysis yields were slightly higher following the harsher optimized acid hydrolysis. This indicates that acid hydrolysis may have a small impact on the enzymatic hydrolysis glucose yields.

*Phanerochaete chrysosporium* and *Lactobacillus plantarum* again showed the greatest glucose yields from enzymatic hydrolysis. This indicates that these organisms are superior in increasing the porosity of cellulose fibers, thereby providing a greater access to enzymes. Additionally, they also facilitate the greater overall breakdown of DDGS component matrices, allowing for greater larval digestion. Micronization by BSFL during the larval digestion period may not contribute a large degree to reducing the crystallinity of cellulose fibers, but a reduction in the particle size greatly increases the surface area which enables for greater glucose yields by enzymatic hydrolysis. Solid state fermentation is shown here to be additive in increasing the degradation of DDGS and subsequent enzymatic hydrolysis glucose yields during the larval digestion period as compared to the control sample.

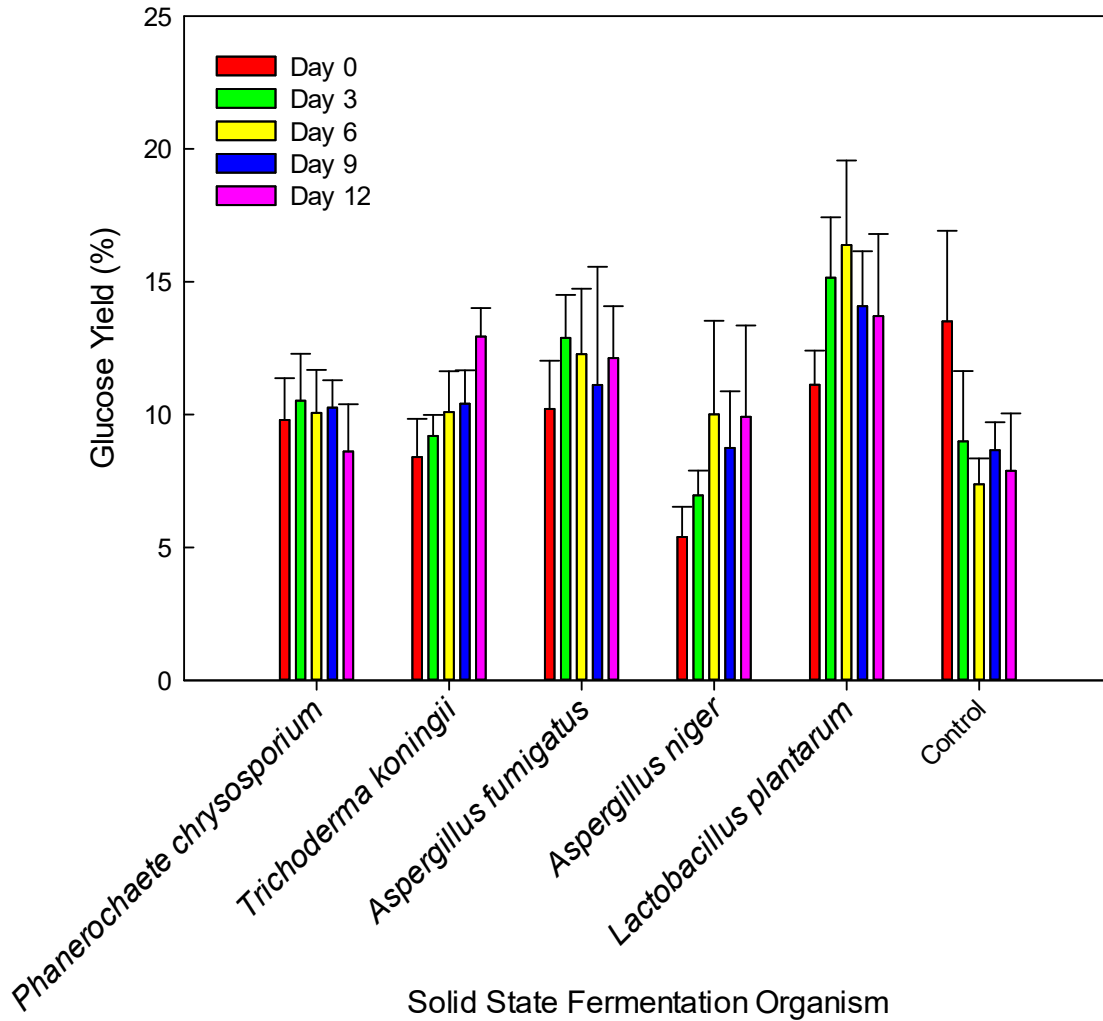
Non-optimized glucose yields were between 4-10 % of the weight of carbohydrates in DDGS. Optimization of the acidic hydrolysis step increased the glucose yields to 5-16% of the carbohydrates, and increased them in every sample as seen on **Fig. 5-34**. When comparing larval digestion periods (day 3 to day 12) across samples, SSF and larval digestion treatment serves to increase the glucose yields compared to the control sample in nearly every case. *Aspergillus niger* is the exception, and was nearly identical to the control. As shown in section 5.3.2., *A. niger* produced high amounts of protease and comparable amounts of cellulase compared to other SSF organisms. This resulted in lower amounts of cellulose being present in DDGS fermented with *A. niger* than other organisms except *T. koningii*, but the CI of the DDGS wasn't altered very much. Low cellulose content in the *A. niger* sample could explain the lack of glucose production under optimized conditions. *Lactobacillus plantarum* and larval digestion had the greatest effect on glucose yields, owing to a large acidic glucose yield.



**Fig. 5-33 Optimized Sugar Liberation by Enzymatic Hydrolysis of Spent Feed from SSF+Larvae during 12 Day Larval Digestion**

Glucose % = mg glucose/(mg dry wt. of substrate \* 0.507) \* 100%; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Enzymatic hydrolysis is preceded by dilute acid hydrolysis which removes structurally weak glucose polymers and free-glucose. Glucose liberation via enzymatic hydrolysis (cellulase) is therefore a measure of the amount of sugars which can be recovered from crystalline cellulose particles under the current system. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the solid state fermentation (SSF) period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation. N = 3; means followed by different letters are significantly different at  $\alpha = 0.05$  by Fisher's LSD within groupings; for *Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus fumigatus*, *Aspergillus niger*, *Lactobacillus plantarum* p = 0.051, > 0.05, > 0.05, > 0.05, = 0.019, > 0.05 respectively and LSD = 1.10, N/A, N/A, N/A, 1.63, N/A respectively;

These optimized acidic hydrolysis conditions helped to make up for deficiencies in SSF treatments in regards to total sugar yields. The total sugar yields for *Trichoderma koningii* and *Aspergillus fumigatus* were now greater than those of *Phanerochaete chrysosporium* as shown



**Fig. 5-34 Optimized Sugar Yield of Spent Feed of SSF+Larvae during 12 Day Larval Digestion**

Glucose % =  $\text{mg glucose} / (\text{mg dry wt. of substrate} * 0.507) * 100\%$ ; 0.507 is the decimal percentage of carbohydrates in untreated DDGS; Combined sugar liberation is a combination of the free-glucose, and yields from both acidic and enzymatic hydrolysis of glucose. Error bars are added std. dev. from each analysis. Untreated dried distiller's grains with solubles (DDGS) is DDGS prior to the solid state fermentation (SSF) period. The day of the sample represents time elapsed since inoculation of the substrate with larvae. Day 0 sample is DDGS after the SSF period, prior to larval inoculation.

on **Fig. 5-34**. This was not the case in the non-optimized sugar hydrolysis as shown on **Fig. 5-30**. SSF and larval digestion still had a positive effect on glucose yields under optimized conditions as compared to the control. And optimized conditions for acidic hydrolysis helped to increase the total glucose yields for every sample, except for *Aspergillus niger*. *Lactobacillus plantarum* is the best organism to use for SSF applications of DDGS when using BSFL, due to the superior glucose yields produced from both acidic and enzymatic hydrolysis under optimized conditions.

### 5.3.5 Proximate Analysis of DDGS and Larvae from SSF and Larval Digestion

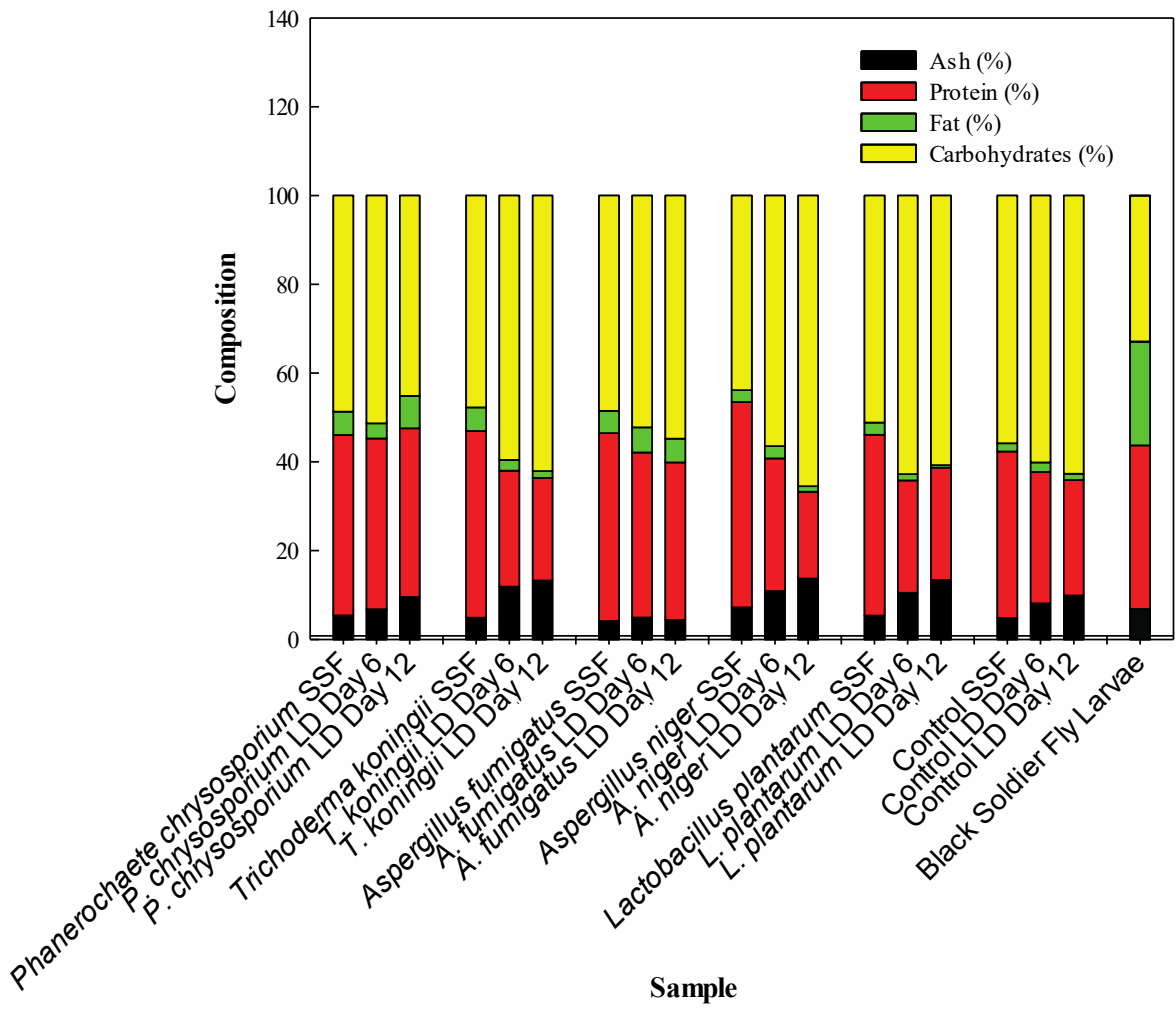
Proximate analysis results for SSF (day 0 of SSF samples), DDGS (Control day 0), SSF and BSFL digested DDGS, and BSFL are shown on **Fig. 5-35**. Ash content is relatively unchanged by SSF staying around 5%, but is heavily increased during larval digestion. It reaches its highest, around 13%, by day 12 of larval digestion for *Trichoderma koningii*, *Aspergillus niger*, and *Lactobacillus plantarum*. Larval ash content is higher than DDGS at around 7%. The ash content increase during larval digestion can be attributed partially to a decrease in the relative concentrations of other components through larval respiration, and incorporation of the organics into larval biomass. The larvae secrete calcium carbonate, a calcium rich substance through their larval integument which helps to harden their exoskeleton. As the larvae molt through instars, they shed their hardened exoskeletons, called larval castings. As these castings become more prevalent in the feed bed of DDGS, the ash content rises, further explaining ash content increases during larval digestion.

SSF has the effect of increasing the protein content and reducing the carbohydrate content of DDGS. This is seen by comparing the day 0 control sample to the day 0 for the SSF treated samples. This proves the second hypothesis of this research that SSF will improve the nutrient profile and increase the digestibility of DDGS. Carbohydrates make up over half of the weight of DDGS at 56%, compared to BSFL which contains only 33% carbohydrates. The reduction of carbohydrate during SSF is due to a decrease in the fiber content of DDGS. *Aspergillus niger* fermentation resulted in the greatest reduction of DDGS carbohydrates which had a day 0 carbohydrate concentration of 43%. Larval digestion increased the carbohydrate content over the 12-day digestion period for every sample except *Phanerochaete chrysosporium*. The decrease in carbohydrates was less than the increase in cellulose content over the larval digestion period indicating a concurrent reduction in other carbohydrates, presumably starch and

hemicelluloses. *Phanerochaete chrysosporium* and *Lactobacillus plantarum* had a slight carbohydrate decrease from days 6 to 12 of larval digestion. These samples also resulted in the greatest total glucose yields under acidic and enzymatic hydrolysis for both non-optimized and optimized conditions. This indicates that these samples have superior degradative properties to the DDGS component matrices. SSF and BSFL digestion resulted in lower day 12 carbohydrate concentrations compared to the control except in the case of *Aspergillus niger*, and *Trichoderma koningii*. The latter of which had equal carbohydrate concentrations to the control. This confirms that SSF in general is an effective way to increase utilization of DDGS carbohydrates.

The protein content of DDGS and BSFL are both high at 38 and 37% respectively. SSF increases this for every sample between 2-5%, except for *Aspergillus niger* which increases it by 8% to 46%. The increase in proteins following SSF is due to the production of fungal biomass, or bacterial protein in the case of *L. plantarum*. Larval digestion reduces the protein content of DDGS. This decrease was the greatest for *Aspergillus niger* treated sample, which had a final protein concentration of 20%. BSFL, which are shown here to have a high protein content, digest the DDGS proteins using them for metabolism and incorporation into larval biomass.

The fat content of DDGS was low at 2%, compared to BSFL which have a fat content of 23%. The fat content of BSFL is dependent upon the feed source and is highly variable ranging from 15-50%<sup>108</sup>. Therefore BSFL fat contents in the present are closer to the lower end of the range for BSFL. This may be the result of feeding them low fat DDGS. SSF increased the fat content for every sample to 3-5%, while larval digestion had no impact on fat contents. The fat content did not have a consistent trend over the larval digestion period. The fat content of BSFL is relatively low here compared to most literature values. DDGS has a low fat content, and likely does not supply optimal levels of fat to support high fat larval biomass creation.



**Fig. 5-35 Proximate Analysis of DDGS and Larvae from SSF and Larval Digestion**

The days in the sample labels represent the time elapsed since inoculation of solid state fermented (SSF) dried distiller's grains with solubles (DDGS) with Black Soldier Fly larvae (BSFL). Day 0 for each sample represents the substrate after SSF, but prior to the inoculation with BSFL. The control sample underwent an identical, but sterile SSF treatment. The effect of SSF can be seen by comparing day 0 for SSF samples to day 0 of the control.

## 6 SUMMARY AND CONCLUSIONS

DDGS, a by-product of grain ethanol fermentation, contains large amounts of protein, resistant starch, and cellulose. The recovery of glucose from the hydrolysis of that starch and cellulose represents an attractive way to increase the total glucose, and thereby ethanol, yields from the starting grains in bioethanol production. This helps to alleviate food security, increase energy independence, and improve the economy of bioethanol production. Glucose recovery is possible through dilute acid and enzymatic hydrolysis, although this process complicates protein recovery. Since the competitiveness of biofuel much depends on the yields of high value commodities from the inputs. In the current practise, the DDGS is utilized as the feedstuff which is not considered as a high value product. Thus additional recovery of valuable substances, such as proteins, is a critical factor that should coexist with the sugar recovery. BSFL are well suited towards agricultural scale production, and are capable of living on and converting DDGS into high protein, high fat larval biomass. BSFL bioconversion using DDGS yields high protein and fat biomass and concentrates carbohydrates in DDGS, greatly increasing value recovery from the biofuel byproduct.

Despite its benefits, it was shown that BSFL also have less digestive abilities with higher cellulose content feeds. Results from this research showed that the use of SSF, which is a low energy and food-safe process, facilitates the degradation of DDGS by BSFL digestion through the secretion of extracellular enzymes, notably cellulase. It also increases the protein concentration of DDGS.

Study 1 proves the first hypothesis of this research, that the concentration of enzymes in spent larval feed is dependent upon the composition of the starting feed. SSF was then shown to improve the nutrient profile and increase the digestibility of DDGS, as well as increase sugar liberation, thereby proving the second and third hypothesis respectively. Finally, the fourth hypothesis, that BSFL action is sufficient to increase sugar liberation in wheat DDGS, was disproven.

The following objectives were achieved: 1) testing enzyme activity of spent larval feeds, 3) evaluate cellulose crystallinity changes from BSFL and SSF treatments, 4) to measure sugar liberation changes from BSFL and SSF treatments, and 5) to quantify changes to DDGS during processing by proximate analysis; objective 2) to determine feed consumption rates and bioconversion was not fully realized, but efforts have led to further research opportunities.

Together, SSF and subsequent BSFL digestion of DDGS is an efficient way to recover DDGS proteins and fermentable glucose which proves the overall hypothesis of this research. In addition to improving the total glucose yield from starting grains, this process helps to further commercialize DDGS and insect biomass. Insect biomass represents a burgeoning agricultural field which is set to revolutionize the agricultural and aquaculture industries. Finally, the creation of multiple product streams from DDGS bioprocessing gives DDGS robustness against commodity prices and improves the economy of this process.



## 7 PROSPECTIVE RESEARCH

For further research, characterization of the specific enzymes produced by the target SSF organisms needs to be assayed, and changes to DDGS structures more thoroughly quantified. A greater scientific understanding of the parameters which effect SSF, particularly for *Phanerochaete chrysosporium* would allow for greater utilization of this organism in delignification processes for lignocellulosic biomass. In order to fully utilize these organisms as a pretreatment step prior to BSFL digestion, a greater understanding of fungal infections of larvae is needed to prevent retardation of larval growth, and to maximize the survivorship particularly of young larvae. Given that previous research has shown that *L. plantarum* is capable of co-SSF with certain fungal organisms, trails involving such co-fermentation are needed. Additionally, co-fermentations using combinations of the fungal organisms used in the current research may prove useful and should be evaluated. These co-fermentations may enhance the nutritive properties of DDGS above and beyond a monoculture involving either bacterial or fungal fermentation organisms. One of the notable changes to DDGS during SSF is to the lignocellulose itself, which is made primarily of hemicellulose. Further research is needed to quantify change in liberation of monomeric sugars from dilute acid hydrolysis of DDGS. An evaluation of the differences in bioconversion, fermentation, and sugar liberation of other biomass materials would also be of interest, particularly corn DDGS due to its similar yet distinct characters to wheat DDGS. Finally, research is needed on the palatability of DDGS fed BSFL in animal feeding trials, particularly for aquaculture and poultry operations as these represent the largest potential markets for BSFL protein meal.

## 8 REFERENCES CITED

1. Spatari S, Zhang Y, Maclean HL. Life Cycle Assessment of Switchgrass- and Corn Stover-Derived Ethanol-Fueled Automobiles. *Environ Sci Technol*. 2005;39:9750–9758.
2. Sheehan J, Himmel M. Enzymes, Energy, and the Environment: A Strategic Perspective on the U.S. Department of Energy's Research and Development Activities for Bioethanol. *Biotechnol Prog*. 1999;15(5):817–827. doi:10.1021/bp990110d.
3. Kim S, Dale BE. Life Cycle Assessment of Various Cropping Systems Utilized for Producing Biofuels: Bioethanol and Biodiesel. *Biomass and Bioenergy*. 2005;29(6):426–439. doi:10.1016/j.biombioe.2005.06.004.
4. Swana J, Yang Y, Behnam M, Thompson R. An analysis of net energy production and feedstock availability for biobutanol and bioethanol. *Bioresour Technol*. 2011;102(2):2112–7. doi:10.1016/j.biortech.2010.08.051.
5. Cardona C a, Sánchez OJ. Fuel Ethanol Production: Process Design Trends and Integration Opportunities. *Bioresour Technol*. 2007;98(12):2415–57. doi:10.1016/j.biortech.2007.01.002.
6. Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G. Bio-Ethanol--The Fuel of Tomorrow from the Residues of Today. *Trends Biotechnol*. 2006;24(12):549–56. doi:10.1016/j.tibtech.2006.10.004.
7. Sánchez OJ, Cardona CA. Trends in Biotechnological Production of Fuel Ethanol from Different Feedstocks. *Bioresour Technol*. 2008;99(13):5270–95. doi:10.1016/j.biortech.2007.11.013.
8. Zaldivar J, Nielsen J, Olsson L. Fuel Ethanol Production from Lignocellulose: A Challenge for Metabolic Engineering and Process Integration. *Appl Microbiol Biotechnol*. 2001;56(1-2):17–34. doi:10.1007/s002530100624.

9. Lynd L, Wyman C, Gerngross T. Biocommodity Engineering. *Biotechnol Prog.* 1999;15(5):777–793. doi:10.1021/bp990109e.
10. Ethanol Industry Statistics. *Renew Fuels Assoc.* 2013:1. Available at: <http://www.ethanolrfa.org/pages/statistics>.
11. Ballerini D, Desmarquest JP, Pourquié J, Nativel F, Rebeller M. Ethanol production from lignocellulosics: Large scale experimentation and economics. *Bioresour Technol.* 1994;50(1):17–23. doi:10.1016/0960-8524(94)90215-1.
12. Service RF. Is there a road ahead for cellulosic ethanol? *Science.* 2010;329(5993):784–5. doi:10.1126/science.329.5993.784.
13. Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F. New improvements for lignocellulosic ethanol. *Curr Opin Biotechnol.* 2009;20(3):372–80. doi:10.1016/j.copbio.2009.05.009.
14. Roy P, Tokuyasu K, Orikasa T, Nakamura N, Shiino T. A Review of Life Cycle Assessment (LCA) of Bioethanol from Lignocellulosic Biomass. *Japan Agric Res Q.* 2012;46(1):41–57.
15. Marx-Figini M. The Control of Molecular Weight and Molecular Weight-Distribution. In: Brown Jr. RM, ed. *Cellulose and Other Natural Polymer Systems*. New York: Plenum; 1982:243–271.
16. Ljungdahl LG, Eriksson KE. Ecology of Microbial Cellulose Degradation. *Adv Microb Ecol.* 1985;8:237–299.
17. Cowling EB, Brown W. Structural Features of Cellulosic Materials in Relation to Enzymatic Hydrolysis. In: Bould RF, ed. *Cellulases and their Applications*. Washington DC; 1969:125–187.
18. Stone JE, Scallan AM, Donefer E, Ahlgren E. Digestibility as a Simple Function of a Molecule of Similar Size to a Cellulase Enzyme. In: Gould RE, ed. *Cellulases and their Applications*. Washington DC; 1969:219–241.
19. Ladisch M, Hong J, Voloch M, Tsao GT. Cellulase Kinetics. *Basic Life Sci.* 1981;18:55–83.

20. Yamanaka S, Watanabe K, Kitamura N, et al. The Structure and Mechanical Properties of Sheets Prepared from Bacterial Cellulose. *J Mater Sci*. 1989;24(9):3141–3145.
21. Fan LT, Lee Y-H, Beardmore DH. Mechanism of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis. *Biotechnol Bioeng*. 1980;22(1):177–199. doi:10.1002/bit.260220113.
22. Lee YH, Fan LT. Kinetic studies of enzymatic hydrolysis of insoluble cellulose: analysis of the initial rates. *Biotechnol Bioeng*. 1982;24(11):2383–406. doi:10.1002/bit.260241107.
23. Gharpuray MM, Lee YH, Fan LT. Structural modification of lignocellulosics by pretreatments to enhance enzymatic hydrolysis. *Biotechnol Bioeng*. 1983;25(1):157–72. doi:10.1002/bit.260250113.
24. Grethlein HE. The Effect of Pore Size Distribution on the Rate of Enzymatic Hydrolysis of Cellulosic Substrates. *Bio/Technology*. 1985;3(2):155–160. doi:10.1038/nbt0285-155.
25. Grous WR, Converse AO, Grethlein HE. Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. *Enzyme Microb Technol*. 1986;8(5):274–280. doi:10.1016/0141-0229(86)90021-9.
26. Brown Jr RM, Saxena IM. Cellulose biosynthesis: A model for understanding the assembly of biopolymers. *Plant Physiol Biochem*. 2000;38(1-2):57–67. doi:10.1016/S0981-9428(00)00168-6.
27. Atalla RH, Hackney JM, Uhlin I, Thompson NS. Hemicelluloses as structure regulators in the aggregation of native cellulose. *Int J Biol Macromol*. 1993;15(2):109–112. doi:10.1016/0141-8130(93)90007-9.
28. Pizzi A, Eaton N. The Structure of Cellulose by Conformational Analysis. 2. The Cellulose Polymer Chain. *J Macromol Sci Part A - Chem*. 1985;22(1):105–137. doi:10.1080/00222338508063300.
29. Blouin F a., Martin LF, Rowland SP. Gel-Permeation Properties of Cellulose: Part III: Measurement of Pore Structure of Unmodified and of Mercerized Cottons in Fibrous Form. *Text Res J*. 1970;40(9):809–813. doi:10.1177/004051757004000908.
30. Cowling EB. Physical and Chemical Constraints in the Hydrolysis of Cellulose and Lignocellulosic Materials. *Biotechnol Bioeng*. 1975;5:163–181.

31. Walker LP, Wilson DB. Enzymatic Hydrolysis of Cellulose : An Overview. *Bioresour Technol.* 1991;36:3–14.
32. Stone JE, Scallan AM. A Structural Model for the Cell Wall of Water-Swollen Wood Pulp Fibres Based on their Accessibility to Macromolecules. *Cellul Chem Technol.* 1968;3:343–358.
33. Peitersen N, Medeiros J, Mandels M. Adsorption of Trichoderma cellulase on cellulose. *Biotechnol Bioeng.* 1977;19(7):1091–4. doi:10.1002/bit.260190710.
34. Ooshima H, Sakata M, Harano Y. Adsorption of cellulase from Trichoderma viride on cellulose. *Biotechnol Bioeng.* 1983;25(12):3103–14. doi:10.1002/bit.260251223.
35. Fan LT, Lee YH. Kinetic studies of enzymatic hydrolysis of insoluble cellulose: Derivation of a mechanistic kinetic model. *Biotechnol Bioeng.* 1983;25(11):2707–33. doi:10.1002/bit.260251115.
36. Bertran MS, Dale BE. Enzymatic hydrolysis and recrystallization behavior of initially amorphous cellulose. *Biotechnol Bioeng.* 1985;27(2):177–81. doi:10.1002/bit.260270212.
37. Segal L, Creely JJ, Martin AE, Conrad CM. An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. *Text Res J.* 1959;29:786–793.
38. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiol Mol Biol Rev.* 2002;66(3):506–577. doi:10.1128/MMBR.66.3.506.
39. Wilkie KCB. Hemicellulose. *Chemtech.* 1983;13(5):306–319.
40. Wong KK, Tan LU, Saddler JN. Multiplicity of Beta-1,4-Xylanase in Microorganisms: Functions and Applications. *Microbiol Rev.* 1988;52(3):305–17. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=373146&tool=pmcentrez&rendertype=abstract>. Accessed December 10, 2013.
41. Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Lukasik R. Hemicelluloses for Fuel Ethanol: A Review. *Bioresour Technol.* 2010;101(13):4775–800. doi:10.1016/j.biortech.2010.01.088.

42. Dien BS, Cotta M a, Jeffries TW. Bacteria engineered for fuel ethanol production: current status. *Appl Microbiol Biotechnol.* 2003;63(3):258–66. doi:10.1007/s00253-003-1444-y.
43. Hahn-Hägerdal B, Karhumaa K, Jeppsson M, Gorwa-Grauslund MF. Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. *Adv Biochem Eng Biotechnol.* 2007;108:147–77. doi:10.1007/10\_2007\_062.
44. Jeffries TW. Engineering the *Pichia stipitis* Genome for Fermentation of Hemicellulose Hydrolysates. In: Wall JD, Hardwood CS, Demain A, eds. *Bioenergy*. Washington DC: ASM Press; 2008:37–47.
45. Sonderegger M, Schumperli M, Sauer U. Metabolic Engineering of a Phosphoketolase Pathway for Pentose Catabolism in *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 2004;70(5):2892–2897. doi:10.1128/AEM.70.5.2892-2897.2004.
46. Kuyper M, Toirkens MJ, Diderich J a, Winkler A a, van Dijken JP, Pronk JT. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res.* 2005;5(10):925–34. doi:10.1016/j.femsyr.2005.04.004.
47. Sauer U. Evolutionary Engineering of Industrially Important Microbial Phenotypes. *Adv Biochem Eng Biotechnol.* 2001;73:129–169.
48. Sonderegger M, Sauer U. Evolutionary Engineering of *Saccharomyces cerevisiae* for Anaerobic Growth on Xylose. *Appl Environ Microbiol.* 2003;69(4):1990–1998. doi:10.1128/AEM.69.4.1990-1998.2003.
49. Wisselink HW, Toirkens MJ, Wu Q, Pronk JT, van Maris AJ a. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. *Appl Environ Microbiol.* 2009;75(4):907–14. doi:10.1128/AEM.02268-08.
50. Lee J. Biological Conversion of Lignocellulosic Biomass to Ethanol. *J Biotechnol.* 1997;56(1):1–24. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9246788>.
51. Hsu TA, Ladisch MR, Tsao GT. Alcohol from Cellulose. *Chem Technol.* 1980;10(5):315–319.

52. McMillan JD. Pretreatment of Lignocellulosic Biomass. In: Himmel ME, Baker JO, Overend RP, eds. *Enzymatic Conversion of Biomass for Fuels Production*. Washington DC: ACS Symp Ser.; 1994:292–234.
53. Hsu TA. Pretreatment of Biomass. In: Wyman CE, ed. *Handbook on Bioethanol, Production and Utilization*. Washington DC: Taylor & Francis; 1996:179–212.
54. Claassen PAM, van Lier JB, Lopez Contreras AM, et al. Utilisation of Biomass for the Supply of Energy Carriers. *Appl Microbiol Biotechnol*. 1999;52(6):741–755. doi:10.1007/s002530051586.
55. Council NR. *Biobased Industrial Products: Research and Commercialization Priorities*. The National Academies Press; 2000. Available at: [http://www.nap.edu/openbook.php?record\\_id=5295](http://www.nap.edu/openbook.php?record_id=5295).
56. Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresour Technol*. 2000;74(1):25–33. doi:10.1016/S0960-8524(99)00161-3.
57. Wyman CE. BIOMASS ETHANOL: Technical Progress, Opportunities, and Commercial Challenges. *Annu Rev Energy Environ*. 1999;24(1):189–226. doi:10.1146/annurev.energy.24.1.189.
58. Chandra RP, Bura R, Mabee WE, Berlin A, Pan X, Saddler JN. Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics? *Adv Biochem Eng Biotechnol*. 2007;108:67–93. doi:10.1007/10\_2007\_064.
59. Brandon SK, Eiteman MA, Patel K, et al. Hydrolysis of Tifton 85 bermudagrass in a pressurized batch hot water reactor. *J Chem Technol Biotechnol*. 2008;83(4):505–512. doi:10.1002/jctb.1824.
60. Dien BS, Li X-L, Iten LB, et al. Enzymatic saccharification of hot-water pretreated corn fiber for production of monosaccharides. *Enzyme Microb Technol*. 2006;39(5):1137–1144. doi:10.1016/j.enzmictec.2006.02.022.
61. Negro MJ, Manzanares P, Ballesteros I, Oliva JM, Cabañas A, Ballesteros M. Hydrothermal Pretreatment Conditions to Enhance Ethanol Production from Poplar Biomass. *Appl Biochem Biotechnol*. 2003;105(1-3):87–100. doi:10.1385/ABAB:105:1-3:87.

62. Rogalinski T, Ingram T, Brunner G. Hydrolysis of lignocellulosic biomass in water under elevated temperatures and pressures. *J Supercrit Fluids*. 2008;47(1):54–63. doi:10.1016/j.supflu.2008.05.003.
63. Zheng Y, Pan Z, Zhang R. Overview of Biomass Pretreatment for Cellulosic Ethanol Production. *Int J Agric Biol Eng*. 2009;2(3):51–69. doi:10.3965/j.issn.1934-6344.2009.03.051-068.
64. Sun Y, Cheng J. Hydrolysis of Lignocellulosic Materials for Ethanol Production: A Review. *Bioresour Technol*. 2002;83(1):1–11. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12058826>.
65. Wenzl HFJ. Chapter IV: The Acid Hydrolysis of Wood. In: *The Chemical Technology of Wood*. New York: Academic Press; 1970:157–252.
66. Hamelinck CN, Hooijdonk G Van, Faaij APC. Ethanol from Lignocellulosic Biomass: Techno-Economic Performance in Short-, Middle- and Long-Term. *Biomass and Bioenergy*. 2005;28(4):384–410. doi:10.1016/j.biombioe.2004.09.002.
67. Ojumu T V, Ogunkunle OA. Production of Glucose from Lignocellulosic under Extremely Low Acid and High Temperature in Batch Process, Auto-hydrolysis Approach. *J Appl Sci*. 2005;5(1):15–17.
68. Coughlin MP, Ljungdahl LG. Comparative Biochemistry of Fungal and Bacterial Cellulolytic Enzyme Systems. In: Aubert J-P, Beguin P, Millet J, eds. *Biochemistry and Genetics of Cellulose Degradation*. New York; 1988:11–30.
69. Zhang Y-HP, Lynd LR. Toward an Aggregated Understanding of Enzymatic Hydrolysis of Cellulose: Noncomplexed Cellulase Systems. *Biotechnol Bioeng*. 2004;88(7):797–824. doi:10.1002/bit.20282.
70. Ladisch MR, Lin KW, Voloch M, Tsao GT. Process considerations in the enzymatic hydrolysis of biomass. *Enzyme Microb Technol*. 1983;5(2):82–102. doi:10.1016/0141-0229(83)90042-X.
71. Breznak JA, Brune A. Role of Microorganisms in the Digestion of Lignocellulose by Termites. *Annu Rev Entomol*. 1994;39:453–487.
72. Woodward J. Synergism in Cellulase Systems. *Bioresour Technol*. 1991;36(1):67–75.



doi:10.1016/0960-8524(91)90100-X.

73. Gilbert HJ, Hazlewood GP. Bacterial Cellulases and Xylanases. *J Gen Microbiol.* 1993;139(2):187–194.
74. Gilkes NR, Henrissat B, Kilburn DG, Miller Jr. RC, Warren RAJ. Domains in Microbial Beta-1,4-Glycanases: Sequence Conservation, Function, and Enzyme Families. *Microbiol Rev.* 1991;55(2):303–315.
75. Mandels M, Reese ET. Inhibition of Cellulases. *Annu Rev Phytopathol.* 1965;3(1):85–102. doi:10.1146/annurev.py.03.090165.000505.
76. Ladisch MR, Gong C-S, Tsao GT. Cellobiose hydrolysis by endoglucanase (glucan glucanhydrolase) from *Trichoderma reesei*: Kinetics and mechanism. *Biotechnol Bioeng.* 1980;22(6):1107–1126. doi:10.1002/bit.260220602.
77. Marsden WL, Gray PP. Enzymatic Hydrolysis of Cellulose in Lignocellulosic Materials. *CRC Crit Rev Biotechnol.* 1986;3(3):235–276.
78. Wilson DB. Cellulases of *Thermomonospora fusca*. *Methods Enzymol.* 1998;160:314–323.
79. Miettinen-Oinonen A, Suominen P. Enhanced Production of *Trichoderma reesei* Endoglucanases and Use of the New Cellulase Preparations in Producing the Stonewashed Effect on Denim Fabric. *Appl Environ Microbiol.* 2002;68(8):3956–3964. doi:10.1128/AEM.68.8.3956-3964.2002.
80. Bollók M, Réczey K. Cellulase enzyme production by various fungal strains on different carbon sources. *Acta Aliment.* 2000;29(2):154–168. doi:10.1556/AAlim.29.2000.2.6.
81. Juhasz T, Kozma K, Szengyel Z. Production of beta glucosidase in mixed culture of *Aspergillus niger* BKMF 1305 and *Trichoderma reesei* RUT-C30. *Food Technol Biotechnol.* 2003;41(1):49–53.
82. Bansal N, Tewari R, Soni R, Soni SK. Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. *Waste Manag.* 2012;32(7):1341–6. doi:10.1016/j.wasman.2012.03.006.
83. Singh D, Chen S. The white-rot fungus *Phanerochaete chrysosporium*: Conditions for the

- production of lignin-degrading enzymes. *Appl Microbiol Biotechnol*. 2008;81(3):399–417. doi:10.1007/s00253-008-1706-9.
84. Dosoretz CG, Dass SB, Reddy C a., Grethlein HE. Protease-mediated degradation of lignin peroxidase in liquid cultures of *Phanerochaete chrysosporium*. *Appl Environ Microbiol*. 1990;56(11):3429–3434.
  85. Kim E, Irwin D, Walker L, Wilson D. Factorial Optimization of a Six-Cellulase Mixture. *Biotechnol Bioeng*. 1998;58(5):494–501. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10099285>.
  86. Zhang N, Li D, Zhang X, Shi Y, Wang H. Solid-state fermentation of whole oats to yield a synbiotic food rich in lactic acid bacteria and prebiotics. *Food Funct*. 2015;6(8):2620–2625. doi:10.1039/C5FO00411J.
  87. Starzyńska-Janiszewska A, Stodolak B, Mickowska B. Effect of controlled lactic acid fermentation on selected bioactive and nutritional parameters of tempeh obtained from unhulled common bean (*Phaseolus vulgaris*) seeds. *J Sci Food Agric*. 2014;94(2):359–366. doi:10.1002/jsfa.6385.
  88. Alterthum F, Ingram L. lactose , and xylose by recombinant Efficient Ethanol Production from Glucose , Lactose , and Xylose by Recombinant *Escherichia colit*. *Appl Environ Microbiol*. 1989;55(8):1943–1948.
  89. Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO. Metabolic Engineering of *Klebsiella oxytoca* M5A1 for Ethanol Production from Xylose and Glucose. *Appl Environ Microbiol*. 1991;57(10):2810–2815.
  90. Lynd LR. Overview and Evaluation of Fuel Ethanol from Cellulosic Biomass: Technology, Economics, the Environment, and Policy. *Annu Rev Energy Environ*. 1996;21(1):403–465. doi:10.1146/annurev.energy.21.1.403.
  91. Gulati M, Westgate PJ, Brewer M, Hendrickson R, Ladisch MR. Sorptive recovery of dilute ethanol from distillation column bottoms stream. *Appl Biochem Biotechnol*. 1996;57-58(1):103–119. doi:10.1007/BF02941692.
  92. Ladisch MR, Voloch M, Hong J, Bienkowski P, Tsao GT. Cornmeal adsorber for dehydrating ethanol vapors. *Ind Eng Chem Process Des Dev*. 1984;23(3):437–443. doi:10.1021/i200026a005.

93. Ladisch MR, Dyck K. Dehydration of ethanol: new approach gives positive energy balance. *Science*. 1979;205(4409):898–900. doi:10.1126/science.205.4409.898.
94. Liu K. Chemical composition of distillers grains, a review. *J Agric Food Chem*. 2011;59(5):1508–1526. doi:10.1021/jf103512z.
95. Li J, Vasanthan T, Gao J, Naguleswaran S, Zijlstra RT, Bressler DC. Resistant Starch Escaped from Ethanol Production: Evidence from Confocal Laser Scanning Microscopy of Distiller’s Dried Grains with Solubles (DDGS). *Cereal Chem*. 2014;91(2):130–138. doi:10.1094/CCHEM-05-13-0087-R.
96. Tunc MS, Van Heiningen ARP. Hemicellulose extraction of mixed southern hardwood with water at 150 °C: Effect of time. *Ind Eng Chem Res*. 2008;47(18):7031–7037. doi:10.1021/ie8007105.
97. Xu Y, Hanna M a. Optimum conditions for dilute acid hydrolysis of hemicellulose in dried distillers grains with solubles. *Ind Crops Prod*. 2010;32(3):511–517. doi:10.1016/j.indcrop.2010.06.024.
98. Nouredini H, Byun J. Dilute-acid pretreatment of distillers’ grains and corn fiber. *Bioresour Technol*. 2010;101(3):1060–1067. doi:10.1016/j.biortech.2009.08.094.
99. May BM. The Occurrence in New Zealand and the Life History of the Soldier Fly *Hermetia illucens* (L.) (Diptera: Stratiomyidae). *New Zeal J Sci Wellingt*. 1961;4:55–65.
100. Bradley SW, Sheppard DC. Housefly Oviposition Inhibition by Larvae of *Hermetia illucens*, the Black Soldier Fly. *J Chem Ecol*. 1984;10(6):853–859.
101. Jong-Gill K, Choi Y-C, Choi J-Y, et al. Ecology of the Black Soldier Fly, *Hermetia illucens* (Diptera: Stratmyidae) in Korea. *Korean J Appl Entomol*. 2008;47(4):337–343.
102. James MT. The Genus *Hermetia* in the United States (Deptera: Stratiomyidae). *Bull Brooklyn Entomol Soc*. 1935;30(4):165–170.
103. Sheppard DC, Tomberlin JK, Joyce JA, Kiser BC, Sumner SM. Rearing Methods for the Black Soldier Fly (Diptera: Stratiomyidae). *J Med Entomol*. 2002;39(4):695–698.
104. Tomberlin JK, Sheppard DC, Joyce JA. Selected Life-History Traits of Black Soldier Flies (Diptera: Stratiomyidae) Reared on Three Artificial Diets. *Ann Entomol Soc Am*.

- 2002;95(3):379–386. doi:10.1603/0013-8746(2002)095[0379:SLHTOB]2.0.CO;2.
105. Li Q, Cai H, Yu Z, Garza E, Zheng L, Zhou S. From Organic Waste to Biodiesel: Black Soldier Fly, *Hermetia illucens*, Makes it Feasible. *Fuel*. 2011;90(4):1545–1548. doi:10.1016/j.fuel.2010.11.016.
  106. FAO. *The State of World Fisheries and Aquaculture, Opportunities and Challenges*. Rome, Italy; 2014.
  107. Widjastuti T, Wiradimadja R, Rusmana D. The Effect of Substitution of Fish Meal by Black Soldier Fly (*Hermetia illucens*) Maggot Meal in the Diet on Production Performance of Quail (*Coturnix coturnix japonica*). *Sci Pap Ser D Anim Sci*. 2014;57:125–129.
  108. Makkar HPS, Tran G, Heuzé V, Ankers P. State-of-the-art on use of insects as animal feed. *Anim Feed Sci Technol*. 2014;197:1–33. doi:10.1016/j.anifeedsci.2014.07.008.
  109. Zheng L, Li Q, Zhang J, Yu Z. Double the Biodiesel Yield: Rearing Black Soldier Fly Larvae, *Hermetia illucens*, on Solid Residual Fraction of Restaurant Waste After Grease Extraction for Biodiesel Production. *Renew Energy*. 2012;41:75–79. doi:10.1016/j.renene.2011.10.004.
  110. Newton GL, Booram C V, Barker RW, Hale OM. Dried *Hermetia Illucens* Larvae Meal as a Supplement for Swine. *J Anim Sci*. 1977;44:395–400. Available at: <http://www.journalofanimalscience.org/content/44/3/395>.
  111. Prakash M. Plant Eating Insects. In: *Insect Ecology*. Darya Ganj, New Dehli, India: Discovery Publishing House Pvt. Ltd.; 2008:134.
  112. Neis E, Dejong C, Rensen S. The Role of Microbial Amino Acid Metabolism in Host Metabolism. *Nutrients*. 2015;7(4):2930–2946. doi:10.3390/nu7042930.
  113. Axtell RC, Arends JJ. Ecology and Management of Arthropod Pests of Poultry. *Annu Rev Entomol*. 1990;35:101–26. doi:10.1146/annurev.en.35.010190.000533.
  114. Allaby M. *A Dictionary of Ecology*. USA: Oxford University Press; 2006.
  115. Sheppard DC, Larry G, Thompson SA, Savage S. A Value Added Manure Management System Using the Black Soldier Fly. *Bioresour Technol*. 1994;50(1994):275–279.

116. Tomberlin JK, Sheppard DC, Joyce JA. Susceptibility of Black Soldier Fly (Diptera: Stratiomyidae) Larvae and Adults to Four Insecticides. *J Econ Entomol.* 2002;95(3):598–602. doi:10.1603/0022-0493-95.3.598.
117. Zhang J, Huang L, He J, et al. An Artificial Light Source Influences Mating and Oviposition of Black Soldier Flies, *Hermetia illucens*. *J Insect Sci.* 2010;10. Available at: [insectscience.org/10.202](http://insectscience.org/10.202).
118. Tingle FC, Mitchell ER, Copeland WW. The Soldier Fly in Poultry Houses in North Central Florida. *J Georg Entomol Soc.* 1975;10(2):179–183.
119. Copello A. Biology of *H. Illucens*, the Fly of our Beehives. *Rev La SEA.* 1926;1(2):23–26.
120. Tomberlin JK, Sheppard DC. Lekking Behavior of the Black Soldier Fly (Diptera: Stratiomyidae). *Florida Entomol.* 2001;84(4):729–730. Available at: <http://www.jstor.org/stable/3496413> .
121. Toft CA. Population structure and mating system of a desert bee fly (*Lordotus pulchrissimus*; Diptera: Bombyliidae). *Oikos.* 1989;54(3):345–358.
122. Tomberlin JK, Adler PH, Myers HM. Development of the Black Soldier Fly (Diptera : Stratiomyidae) in Relation to Temperature. *Environ Entomol.* 2009;38(3):930–934.
123. Fatchurochim S, Geden CJ, Axtell RC. Filth Fly (Diptera) Oviposition and Larval Development in Poultry Manure of Various Moisture Levels. *J Entomol Sci.* 1989;24(2):224–231.
124. Zheng L, Crippen TL, Holmes L, et al. Bacteria Mediate Oviposition by the Black Soldier Fly, *Hermetia illucens* (L.), (Diptera: Stratiomyidae). *Sci Rep.* 2013;3:2563. doi:10.1038/srep02563.
125. Byrd JH, Butler JF. Effects of temperature on *Chrysomya rufifacies* (Diptera: Calliphoridae) development. *J Med Entomol.* 1997;34(3):353–358.
126. Johannsen OA. Stratiomyiid Larvae and Puparia of the North Eastern States. *J New York Entomol Soc.* 1922;30(4):141–153.

127. Diener S, Zurbrügg C, Tockner K. Conversion of Organic Material by Black Soldier Fly Larvae: Establishing Optimal Feeding Rates. *Waste Manag Res.* 2009;27(6):603–10. doi:10.1177/0734242X09103838.
128. Myers HM, Tomberlin JK, Lambert BD, Kattes D. Development of Black Soldier Fly (Diptera: Stratiomyidae) Larvae Fed Dairy Manure. *Environ Entomol.* 2008;37(1):11–15. doi:10.1603/0046-225X(2008)37[11:DOBSFD]2.0.CO;2.
129. Gobbi P, Martínez-sánchez A, Rojo S. The Effects of Larval Diet on Adult Life-History Traits of the Black Soldier Fly, *Hermetia illucens* (Diptera: Stratiomyidae). *Eur J Entomol.* 2013;110(3):461–468.
130. Ujvari B, Wallman JF, Madsen T, Whelan M, Hulbert a J. Experimental Studies of Blowfly (*Calliphora stygia*) Longevity: A Little Dietary Fat is Beneficial but Too Much is Detrimental. *Comp Biochem Physiol A Mol Integr Physiol.* 2009;154(3):383–8. doi:10.1016/j.cbpa.2009.07.012.
131. Yu G, Cheng P, Chen Y, et al. Inoculating Poultry Manure with Companion Bacteria Influences Growth and Development of Black Soldier Fly (Diptera: Stratiomyidae) Larvae. *Environ Entomol.* 2011;40(1):30–5. doi:10.1603/EN10126.
132. Yuan-Feng C. Research on Feed Characteristics of Bacillus Natto. *Chinese J Anim Nutr.* 2009;21(3):371–378.
133. Harris RS, Bronner F. Phytates, Nutrition, and Food Processing. *Food Technol.* 1953;7(12):21–21.
134. Beard RL, Sands DC. Factors Affecting Degradation of Poultry Manure by Flies. *Environ Entomol.* 1973;2(5):801–806. Available at: <http://www.ingentaconnect.com/content/esa/envent/1973/00000002/00000005/art00014>.
135. Holmes LA, Vanlaerhoven SL, Tomberlin JK. Substrate Effects on Pupation and Adult Emergence of *Hermetia illucens* (Diptera: Stratiomyidae). *Environ Entomol.* 2013;42(2):370–374. Available at: <http://www.bioone.org/doi/full/10.1603/EN12255>.
136. Kim W, Bae S, Park K, et al. Biochemical Characterization of Digestive Enzymes in the Black Soldier Fly, *Hermetia illucens* (Diptera: Stratiomyidae). *J Asia Pac Entomol.* 2011;14(1):11–14. doi:10.1016/j.aspen.2010.11.003.

137. Kudo T. Termite-Microbe Symbiotic System and Its Efficient Degradation of Lignocellulose. *Biosci Biotechnol Biochem.* 2009;73(12):2561–2567. doi:10.1271/bbb.90304.
138. Warnecke F, Luginbühl P, Ivanova N, et al. Metagenomic and Functional Analysis of Hindgut Microbiota of a Wood-Feeding Higher Termite. *Nature.* 2007;450(7169):560–570. doi:10.1038/nature06269.
139. Inoue T, Moriya S, Ohkuma M, Kudo T. Molecular Cloning and Characterization of a Cellulase Gene from a Symbiotic Protist of the Lower Termite, *Coptotermes formosanus*. *Gene.* 2005;349:67–75. doi:10.1016/j.gene.2004.11.048.
140. Savage DC. Microbial Ecology of the Gastrointestinal Tract. *Annu Rev Microbiol.* 1977;31:107–133.
141. Berg RD. The Indigenous Gastrointestinal Microflora. *Trends Microbiol.* 1996;4(11):430–5. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8950812>. Accessed December 10, 2013.
142. Lederberg J, McCray AT. 'Ome Sweet 'Omics--A Genealogical Treasury of Words. *Sci.* 2001;15(7):8.
143. Dasch GA, Weiss E, Chang K-P. Endosymbionts of Insects. In: Krieg NR, ed. *Bergey's Manual of Systematic Bacteriology*. Baltimore: JG Holt; 1984:811–813.
144. Tanada Y, Kaya HK. Associations Between Insects and Nonpathogenic Microorganisms. In: *Insect Pathology*. New York; 1993:12–51.
145. Bignell DE. Introduction to Symbiosis. In: *Termites: Evolution, Sociality, Symbioses, Ecology*. Kluwer Academic Publishers; 2000:189–208.
146. Breznak JA. Ecology of Prokaryotic Microbes in the Guts of Wood- and Litter- Feeding Termites. In: *Termites: Evolution, Sociality, Symbioses, Ecology*. Kluwer Academic Publishers; 2000:209–231.
147. Brune A. Termite Guts: The World's Smallest Bioreactors. *Trends Biotechnol.* 1998;16(1):16–21.
148. Breznak JA. Intestinal Microbiota of Termites and other Xylophageous Insects. *Annu Rev*

- Microbiol.* 1982;36:323–343.
149. Dillon RJ, Dillon VM. The Gut Bacteria of Insects: Nonpathogenic Interactions. *Annu Rev Entomol.* 2004;49(98):71–92. doi:10.1146/annurev.ento.49.061802.123416.
  150. Dillon R, Charnley K. Mutualism Between the Desert Locust *Schistocerca gregaria* and its Gut Microbiota. *Res Microbiol.* 2002;153(8):503–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12437211>. Accessed December 10, 2013.
  151. Kaufman M. The Contribution of Hindgut Bacteria to Dietary Carbohydrate Utilization by Crickets (Orthoptera: Gryllidae). *Comp Biochem Physiol A- Physiol.* 1991;98(1):117–123.
  152. Santo Domingo JW, Kaufman MG, Klug MJ, Holben WE, Harris D, Tiedje JM. Influence of Diet on the Structure and Function of the Bacterial Hindgut Community of Crickets. *Mol Ecol.* 1998;7(6):761–767. doi:10.1046/j.1365-294x.1998.00390.x.
  153. Douglas AE. Microbial Brokers of Insect-Plant Interactions. *Ser Entomol.* 1992;49:329–336.
  154. Jones CG. Microorganisms as Mediators of Plant Resource Exploitation by Insect Herbivores. In: Price PW, Slobodchikoff CN, Gaud WS, eds. *A New Ecology: Novel Approaches to Interactive Systems*. New York; 1984:53–99.
  155. Bhat TK, Singh B, Sharma OP. Microbial Degradation of Tannins--A Current Perspective. *Biodegradation.* 1998;9(5):343–57. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10192896>. Accessed December 10, 2013.
  156. Brune A, Miambi E, Breznak JA. Roles of Oxygen and the Intestinal Microflora in the Metabolism of Lignin-Derived Phenylpropanoids and other Monoaromatic Compounds by Termites. *Appl Environ Microbiol.* 1995;61(7):2688–2695.
  157. Terra WR, Ferreira C, Jordao BP, Dillon RJ. Digestive Enzymes. In: *Biology of the Insect Gut*. London: Chapman & Hall; 1996:153–194.
  158. Park D-S, Oh H-W, Jeong W-J, Kim H, Park H-Y, Bae KS. A Culture-Based Study of the Bacterial Communities Within the Guts of Nine Longicorn Beetle Species and their Exo-Enzyme Producing Properties for Degrading Xylan and Pectin. *J Microbiol.* 2007;45(5):394–401. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17978798>.



159. Olsen GJ, Woese CR, Overbeek R. The Winds of (Evolutionary) Change: Breathing New Life into Microbiology. *J Bacteriol.* 1994;176:1–6.
160. Woese CR. Interpreting the universal phylogenetic tree. *Proc Natl Acad Sci.* 2000;97(15):8392–8396. doi:10.1073/pnas.97.15.8392.
161. Rainey F. Description of *Caldicellulosiruptor saccharolyticus* gen. nov., sp. nov: An obligately anaerobic, extremely thermophilic, cellulolytic bacterium. *FEMS Microbiol Lett.* 1994;120(3):263–266. doi:10.1016/0378-1097(94)90481-2.
162. Strauss J, Mach RL, Zeilinger S, et al. CreI, the carbon catabolite repressor protein from *Trichoderma reesei*. *FEBS Lett.* 1995;376(1-2):103–107. doi:10.1016/0014-5793(95)01255-5.
163. Schwarz WH. The cellulosome and cellulose degradation by anaerobic bacteria. *Appl Microbiol Biotechnol.* 2001;56(5-6):634–649. doi:10.1007/s002530100710.
164. Jeon H, Park S, Choi J, et al. The Intestinal Bacterial Community in the Food Waste-Reducing Larvae of *Hermetia illucens*. *Curr Microbiol.* 2011;62(5):1390–9. doi:10.1007/s00284-011-9874-8.
165. Zheng L, Crippen TL, Singh B, et al. A Survey of Bacterial Diversity From Successive Life Stages of Black Soldier Fly (Diptera : Stratiomyidae ) by using 16S rDNA Pyrosequencing A Survey of Bacterial Diversity From Successive Life Stages of Black Soldier Fly (Diptera : Stratiomyidae ) by U. *J Med Entomol.* 2013;50(3):647–658. Available at: <http://www.bioone.org/doi/full/10.1603/ME12199>.
166. Yu G-H, Niu C-Y, He G-B, Zhou L, Xia Q, Cheng P. Isolation and Identification of Bacteria Producing Enzymes from Gut and Skin of Black Soldier Fly (*Hermetia illucens*) Larvae. *Chinese Bull Entomol.* 2010;47(5):889–894.
167. Čičková H, Newton GL, Lacy RC, Kozánek M. The use of fly larvae for organic waste treatment. *Waste Manag.* 2015;35:68–80. doi:10.1016/j.wasman.2014.09.026.
168. Furman DP, Young RD, Catts EP. *Hermetia illucens* (Linnaeus) as a factor in the natural control of *Musca domestica* Linnaeus. *J Econ Entomol.* 1959;52(5):917–921.
169. Tomberlin JK, Sheppard DC. Factors Influencing Mating and Oviposition of BSFL in a Colony. *J Entomol Sci.* 2002;37(4):345–352.

170. Erickson MC, Islam M, Sheppard DC, Liao J, Doyle MP. Reduction of Escherichia coli O157:H7 and Salmonella enterica Serovar Enteritidis in Chicken Manure by Larvae of the Black Soldier Fly. *J Food Prot.* 2004;4:685–690.
171. Hoffmann J a, Hetru C. Insect Defensins: Inducible Antibacterial Peptides. *Immunol Today.* 1992;13(10):411–5. doi:10.1016/0167-5699(92)90092-L.
172. Liu Q, Tomberlin JK, Brady JA, Sanford MR. Black Soldier Fly (Diptera : Stratiomyidae) Larvae Reduce Escherichia coli in Dairy Manure. *Environ Entomol.* 2008;37(6):1525–1530.
173. Shiau S-Y, Yu Y-P. Dietary supplementation of chitin and chitosan depresses growth in tilapia, *Oreochromis niloticus* × *O. aureus*. *Aquaculture.* 1999;179(1-4):439–446. doi:10.1016/S0044-8486(99)00177-5.
174. Kroeckel S, Harjes A-GE, Roth I, et al. When a Turbot Catches a Fly: Evaluation of a Pre-Pupae Meal of the Black Soldier Fly (*Hermetia illucens*) as Fish Meal Substitute — Growth Performance and Chitin Degradation in Juvenile Turbot (*Psetta maxima*). *Aquaculture.* 2012;364-365:345–352. doi:10.1016/j.aquaculture.2012.08.041.
175. Popa R, Green TR. Using Black Soldier Fly Larvae for Processing Organic Leachates. *J Econ Entomol.* 2012;105(2):374–378. doi:10.1603/EC11192.
176. Green TR, Popa R. Enhanced Ammonia Content in Compost Leachate Processed by Black Soldier Fly Larvae. *Appl Biochem Biotechnol.* 2012;166(6):1381–7. doi:10.1007/s12010-011-9530-6.
177. Berridge MJ. The Physiology of Excretion in the Cotton Stainer, *Dysdercus Fasciatus* Signoret. *J Exp Biol.* 1965;43(3):535–552.
178. Wigglesworth VB. The Physiology Of Excretion In A Blood-Sucking Insect, *Rhodnius Prolixus* (Hemiptera, Reduviidae). *J Exp Biol.* 1931;8(4):443–451.
179. Brown AWA. The Nitrogen Metabolism of an Insect (*Lucilia Seicata* Mg.) II. Ammonia and Other Metabolites. *Biochem J.* 1938;32(1):903–912.
180. Robinson W. Allantoin, a Constituent of Maggot Excretions, Stimulates Healing of Chronic Discharging Wounds. *J Parasitol.* 2013;21(5):354–358. Available at:

<http://www.jstor.org/stable/3271945>.

181. Baer WS. The Treatment of Chronic Osteomyelitis with the Maggot (Larva of the Blowfly). *J Bone Jt Surg*. 1931;13(3):920–944.
182. McLellan NW. The Maggot Treatment of Osteomyelitis. *Can Med Assoc J*. 1932:256–260.
183. Mumcuoglu KY, Ingber A, Gilead L, et al. Maggot Therapy for the Treatment of Intractable Wounds. *Int J Dermatol*. 1999;38(8):623–627.
184. Thomas S, Andrews AM, Hay NP, Bourgoise S. The Anti-Microbial Activity of Maggot Secretions: Results of a Preliminary Study. *J Tissue Viability*. 1999;9(4):127–132.
185. Feng X, Cheng G, Chen S, Yang H, Huang W. Evaluation of the Burn Healing Properties of Oil Extraction from Housefly Larva in Mice. *J Ethnopharmacol*. 2010;130(3):586–92. doi:10.1016/j.jep.2010.05.044.
186. Choi W-H, Yun J-H, Chu J-P, Chu K-B. Antibacterial Effect of Extracts of *Hermetia illucens* (Diptera: Stratiomyidae) Larvae Against Gram-Negative Bacteria. *Entomol Res*. 2012;42(5):219–226. doi:10.1111/j.1748-5967.2012.00465.x.
187. Lalander C, Diener S, Magri ME, Zurbrügg C, Lindström A, Vinnerås B. Faecal Sludge Management with the Larvae of the Black Soldier Fly (*Hermetia illucens*)--From a Hygiene Aspect. *Sci Total Environ*. 2013;458-460:312–8. doi:10.1016/j.scitotenv.2013.04.033.
188. Chu K-B, Jeon G-C, Quan F-S. Hexanedioic acid from *Hermetia illucens* larvae (Diptera: Stratiomyidae) protects mice against *Klebsiella pneumoniae* infection. *Entomol Res*. 2014;44(1):1–8. doi:10.1111/1748-5967.12043.
189. Zhou F, Tomberlin JK, Zheng L, Yu Z, Zhang J. Developmental and Waste Reduction Plasticity of Three Black Soldier Fly Strains (Diptera: Stratiomyidae) Raised on Different Livestock Manures. *J Med Entomol*. 2013;50(6):1224–1230. doi:10.1603/ME13021.
190. Li Q, Zheng L, Qiu N, Cai H, Tomberlin JK, Yu Z. Bioconversion of Dairy Manure by Black Soldier Fly (Diptera: Stratiomyidae) for Biodiesel and Sugar Production. *Waste Manag*. 2011;31(6):1316–20. doi:10.1016/j.wasman.2011.01.005.

191. Wilkie AC, Mulbry WW. Recovery of Dairy Manure Nutrients by Benthic Freshwater Algae. *Bioresour Technol.* 2002;84(1):81–91. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12137274>. Accessed November 24, 2013.
192. Li Q, Hou Y, Li W, Yu Z, Zheng L, Yang S. Biodiesel Production from Rice Straw and Restaurant Waste Employing Black Soldier Fly Assisted by Microbes. *Energy.* 2012;47(1):225–229. doi:10.1016/j.energy.2012.09.006.
193. Lee C-M, Lee Y-S, Seo S-H, et al. Screening and characterization of a novel cellulase gene from the gut microflora of *Hermetia illucens* using metagenomic library. *J Microb Biotechnol.* 2014;24(9):1196–1206.
194. Yang S, Lio J, Wang T. Evaluation of enzyme activity and fiber content of soybean cotyledon fiber and distiller's dried grains with solubles by solid state fermentation. *Appl Biochem Biotechnol.* 2012;167(1):109–21. doi:10.1007/s12010-012-9665-0.
195. Lio J, Wang T. Solid-state fermentation of soybean and corn processing coproducts for potential feed improvement. *J Agric Food Chem.* 2012;60(31):7702–9. doi:10.1021/jf301674u.
196. Schuster E, Dunn-Coleman N, Frisvad J, Van Dijck P. On the safety of *Aspergillus niger* - A review. *Appl Microbiol Biotechnol.* 2002;59(4-5):426–435. doi:10.1007/s00253-002-1032-6.
197. Zhang Y, Caupert J. Survey of mycotoxins in U.S. distiller's dried grains with solubles from 2009 to 2011. *J Agric Food Chem.* 2012;60(2):539–43. doi:10.1021/jf203429f.
198. Worthington V, ed. Protease, *S. Aureus*, V8. In: *Worthington Enzyme Manual : Enzymes and Related Biochemicals*. Lakewood, New Jersey 0870 U.S.A.: Worthington Biochemical Company; 1993:339.
199. Worthington V, ed. Alpha Amylase. In: *Worthington Enzyme Manual : Enzymes and Related Biochemicals*. Lakewood, New Jersey 0870 U.S.A.: Worthington Biochemical Company; 1993:36–41.
200. Worthington V, ed. Lipase. In: *Worthington Enzyme Manual : Enzymes and Related Biochemicals*. Lakewood, New Jersey 0870 U.S.A.: Worthington Biochemical Company; 1993:243–246.

201. Worthington V, ed. Beta Amylase. In: *Worthington Enzyme Manual : Enzymes and Related Biochemicals*. Lakewood, New Jersey 0870 U.S.A.: Worthington Biochemical Company; 1993:42–44.
202. Worthington V, ed. Cellulase. In: *Worthington Enzyme Manual : Enzymes and Related Biochemicals*. Lakewood, New Jersey 0870 U.S.A.: Worthington Biochemical Company; 1993:81–85.
203. Brendel O, Iannetta PPM, Stewart D. A rapid and simple method to isolate pure alpha-cellulose. *Phytochem Anal*. 2000;11(1):7–10. doi:10.1002/(SICI)1099-1565(200001/02)11:1<7::AID-PCA488>3.0.CO;2-U.
204. Singh a. Mechanisms of Reactions of Chlorine, Chlorine Dioxide and Nitrogen-Dioxide. *J Pulp Pap Sci*. 1990;16(2):J48–J53.
205. Hessler LE, Power RE. The Use of Iodine Adsorption as a Measure of Cellulose Fiber Crystallinity. *Text Res J*. 1954;24(9):822–827. doi:10.1177/004051755402400906.
206. Technical A. Crude Fat in Grain and Stock Feeds. In: *AACC International Approved Methods*. AACC International; 2009:1–2. doi:10.1094/AACCIntMethod-30-20.01.
207. *Ash-Basic Method: AACC International Approved Method 08-01.01*. AACC International; 2009. doi:10.1094/AACCIntMethods.
208. Technical A. Crude Protein--Combustion Method. In: *AACC International Approved Methods*. AACC International; 2009:1–2. doi:10.1094/AACCIntMethod-46-30.01.
209. *Full Report (All Nutrients): 20020, Cornmeal, whole-grain, yellow.*; 2014. Available at: <http://ndb.nal.usda.gov/ndb/foods/show/6491?fgcd=&man=&lfacet=&count=&max=&sort=&qlookup=&offset=&format=Full&new=&measureby=>.
210. *Full Report (All Nutrients) 01090, Milk, dry, whole, with added vitamin D.*; 2014. Available at: <http://ndb.nal.usda.gov/ndb/foods/show/83?fgcd=&manu=&lfacet=&format=&count=&max=35&offset=&sort=&qlookup=whole+milk+powder>.
211. University of Saskatchewan's Wheat DDGS Nutrient Profile. 2010. Available at: <http://www.ddgs.usask.ca/NutrientProfile.aspx>. Accessed January 1, 2014.

212. Ryan MP, Adley CC. *Sphingomonas paucimobilis*: A persistent Gram-negative nosocomial infectious organism. *J Hosp Infect.* 2010;75(3):153–157. doi:10.1016/j.jhin.2010.03.007.
213. Park S, Chang BS, Yoe SM. Detection of antimicrobial substances from larvae of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). *Entomol Res.* 2014;44(2):58–64. doi:10.1111/1748-5967.12050.
214. Adav SS, Ravindran A, Sze SK. Quantitative proteomic study of *Aspergillus Fumigatus* secretome revealed deamidation of secretory enzymes. *J Proteomics.* 2015;119:154–168. doi:10.1016/j.jprot.2015.02.007.
215. Wood TM, McCrae SI. The cellulase of *Trichoderma koningii*. Purification and properties of some endoglucanase components with special reference to their action on cellulose when acting alone and in synergism with the cellobiohydrolase. *Biochem J.* 1978;171(1):61–72.