USE OF GENETICALLY MODIFIED *SACCHAROMYCES CEREVISIAE* TO CONVERT SOLUBLE STARCH DIRECTLY TO BIOETHANOL

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By

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ABSTRACT

Ethanol can be used as a complete fuel or as an octane enhancer, and has the advantages of being renewable and environmentally friendly. Ethanol produced by a fermentation process, generally referred to as bioethanol, is considered to be a partial solution to the worldwide energy crisis. Traditionally, industrial bioethanol fermentation involves two major steps: starch hydrolysis and fermentation. Since the key microorganism, *Saccharomyces cerevisiae*, lacks amylolytic activity and is unable to directly utilize starch for proliferation and fermentation, it requires intensive amount of energy and pure starch hydrolyzing enzymes to gelatinize, liquefy and dextrinize the raw starch before fermentation.

It has been suggested that genetically engineered yeast which expresses amylolytic enzymes could potentially perform simultaneous starch hydrolysis and fermentation. This improvement could greatly reduce the capital and energy costs in current bioethanol producing plants and make bioethanol production more economical. In this project, a novel yeast strain of *Saccharomyces cerevisiae* was genetically engineered in such a way that barley α-amylase was constitutively expressed and immobilized on the yeast cell surface. This particular α-amylase was selected based on its superior kinetic properties and its pH optimum which is compatible with the pH of yeast culture media. The cDNA encoding barley α-amylase, with a secretion signal sequence, was fused to the cDNA encoding the C-terminal half of a cell wall anchoring protein, α-agglutinin. The fusion gene was cloned downstream of a constitutive promoter *ADH1* in a yeast episomal plasmid pAMY. The pAMY harbouring yeast showed detectable amylolytic activity in a starch plate assay. In addition, α-amylase activity was detected only in the cell pellet fraction and not in the culture supernatant. In batch fermentation studies using soluble wheat starch as sole carbon source, even though pAMY harbouring yeast was able to hydrolyse soluble starch under fermentation conditions, no ethanol was produced. This was probably due to insufficient α-amylase activity which resulted from the enzyme being anchored on the cell wall by α-agglutinin. Further research using alternative cell surface anchoring system might be able to produce yeast with industrial applications.

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> "Every day I remind myself that my inner and outer life are based on the labours of other men, living and dead, and that I must exert myself in order to give in the same measure as I have received and am still receiving."
>
> - Albert Einstein

To my grandmother
Zhao, lanmeng

TABLE OF CONTENTS

PERMISSION TO USE……………………………………………………………… ……... i

ABSTRACT…………………………………………………………………………….. ii

ACKNOWLEDGEMENTS…………………………………………………………...iv

TABLE OF CONTENTS……………………………………………………………...v

LIST OF TABLES……………………………………………………………………ix

LIST OF FIGURES………………………………………………………………….... x

LIST OF ABBREVIATIONS………………………………………………………xii

1.0 INTRODUCTION………………………………………………………………. 1

2.0 LITERATURE REVIEW……………………………………………………….. 3

2.1 Bioethanol……………………………………………………………………..3

2.2 A Review of the Current Industrial Starch Fermentation Process…………6

2.3 Application of DNA Recombinant Techniques to Make Bioethanol Production More Economically Competitive……………………………… 8

2.3.1 Recombinant Yeast Strains that Secrete Starch Hydrolysing Enzymes……………………………………………………………8

2.3.2 Recombinant Yeast Strains with Cell Surface Anchored Amylolytic Enzymes……………………………………………..11

2.3.2.1 Enzyme Stability……………………………………………11

2.3.2.2 A Cell Wall Anchoring Protein α-Agglutinin and GPI Anchor……………………………………………… 12
2.3.2.3 Recombinant Yeast with Cell Surface Anchored Starch Hydrolysing Enzymes………………………………13
2.3.2.4 Domain Functions and Structure of Barley α-Amylase……………………………………………… 18
2.3.3 Plasmid Stability……………………………………………… 19
2.3.4 Cell Surface Anchored Yeast Expression System vs. Secreted Yeast Expression System………………………………… 23
2.4 Assays Used to Detect and Quantify Amylolytic Activity………………… 23
2.4.1 Starch Plate Assay……………………………………………..23
2.4.2 Iodine Assay……………………………………………………...24
2.4.3 3, 5-Dinitrosalicylic Acid (DNS) Assay…………………………… 24
2.5 Objectives……………………………………………………….. 27

3.0 MATERIALS AND METHODS………………………………………………...28
3.1 Reagents………………………………………………………………….28
3.2 Bacteria Strain, Yeast Strains and Media Preparations…………………… 30
3.3 DNA Sub-Cloning and Plasmid Construction……………………………... 31
3.3.1 Polymerase Chain Reaction (PCR)………………………………… 31
3.3.2 Restriction Digestion of Plasmid DNA and PCR Generated DNA Fragments……………………………………………… 32
3.3.3 Agarose Gel Electrophoresis………………………………………..32
3.3.4 Ligation of Digested Plasmid DNA and PCR DNA Fragments…………………………………………………………...32
3.3.5 Competent Bacteria Preparation…………………………………… 32
3.3.6 *E. coli* Transformation…………………………………………... 33
3.3.7 Mini-Preparation of Plasmid DNA………………………………… 33
3.4 Yeast Transformation and Selection………………………………………..34
3.4.1 Preparation of Competent Yeast…………………………………… 34
3.4.2 Yeast Transformation……………………………………………….34

3.4.3 Determination of the Appropriate Concentration of Blasticidin to Use for Selection………………………………….. 35
3.5 α-Amylase Assays………………………………………………………35
3.5.1 Detection of α-Amylase Activity on Starch Containing YPD Plate Using Iodine Vapour……………………………………….35
3.5.2 Quantification of Cell Surface Anchored α-Amylase Activity by the 3, 5-Dinitrosalicylic Acid (DNS) Assay and the Iodine Assay………………………………………………………...36
3.6 Batch Fermentation……………………………………………………37
3.6.1 Seeding Cultures…………………………………………………37
3.6.2 Bioreactor Conditions and Media Composition…………………… 37
3.6.3 Starch Hydrolysing Enzymes…………………………………….38
3.6.4 Biomass Analysis…………………………………………………...38
3.6.5 Ethanol Analysis…………………………………………………39
3.6.6 Soluble Starch Concentration ……………………………………... 40
3.6.7 Plasmid Stability ………………………………………………….... 40

4.0 RESULTS…………………………………………………………………….. 41
4.1 Construction of Novel Recombinant Plasmid pAMY……………………... 42
4.2 Recombinant Yeast with Cell Surface Anchored Barley α-Amylase………43
4.3 Determination of the Appropriate Concentration of Blasticidin to Use for Selection…………………………………………………………43
4.4 Detection of α-Amylase Activity in Recombinant Yeast Using Iodine Vapour Approach………………………………………………….46
4.5 Quantification of α-Amylase Activity with DNS and Iodine Assays……... 49
4.6 Batch Fermentation Studies…………………………………………….... 55
4.6.1 Batch Fermentation on Soluble Starch…………………………….. 55
4.6.2 Batch Fermentation with Addition of Amylolytic Enzymes………. 58
4.6.3 Measurement of the Starch Concentration in Batch Fermentation Using the Iodine Assay……………………………65

4.6.4 Plasmid Stability of NRRL Y-132/pAMY in Batch Fermentation………………………………………………………….. 68

5.0 DISCUSSION………………………………………………………………….... 74

5.1 Detection and Quantification of α-Amylase Activity in Intact Yeast……... 74

5.2 Effects of Cell Surface Anchoring on α-Amylase Activity………………...77

5.3 Batch Fermentation on Soluble Starch…………………………………….. 78

5.4 Conclusions……………………………………………………………………80

5.5 Future Directions…………………………………………………………... 81

6.0 REFERENCES…………………………………………………………………….. 84

LIST OF TABLES

Table 3.1: Lists of Reagents and Suppliers…………………………………………………28
Table 3.2: List of Commercial Kits……………………………………………….……….. 29
Table 3.3: List of Oligonucleotides (Invitrogen) for PCR…………………………………29
Table 3.4: Names and Addresses of Suppliers…………………………………………...30
Table 4.1: Quantification and Distribution of α-Amylase Activity Using
the DNS Assay……………………………………………………………….54
Table 4.2: Quantification and Distribution of α-Amylase Activity Using the
Iodine Assay………………………………………………………………….54
Table 4.3: Effect of Boiling on Starch Concentration Determination in Frozen
Samples…………………………………………………………………… 67
Table 4.4: Effect of Yeast Media on Starch Concentration Determination………………... 69

LIST OF FIGURES

Figure 2.1: Aerobic and Anaerobic Catabolic Pathways for Glucose Utilization in Yeast…………………………………………………………… 4

Figure 2.2: Structure of Amylose and Amylopectin……………………………………..7

Figure 2.3: Schematic Representation of the Action of Amylases on Starch……………… 9

Figure 2.4: Structure of the Cell Wall of *S. cerevisiae* …………………………………… 14

Figure 2.5: Structure of α-Agglutinin ……………………………………………………15

Figure 2.6: Schematic Representation of α-Agglutinin Localization by the GPI Anchor…….. ………………………………………………………………16

Figure 2.7: Three-Dimensional Structure of Barley α-Amylase Isozyme AMY1………… 20

Figure 2.8: Structure of the Starch-Iodine Complex……………………………………..25

Figure 2.9: Reaction Mechanism of the DNS Assay………………………………………26

Figure 4.1: Structure of the Fusion Gene…………………………………………………...42

Figure 4.2: The Structure of Yeast Expression Plasmid pAMY……………………………44

Figure 4.3: Model of α-Amylase Expressing Recombinant Yeast…………………………45

Figure 4.4: Cell Growth Response to Various Blasticidin Concentrations on YPD Agar……………………………………………………………………47

Figure 4.5: Detection of α-Amylase Activity in NRRL Y-132/pAMY Using the Iodine Vapour Assay……………………………………………………48

Figure 4.6: Iodine Assay and DNS Assay for Measuring Cell Surface Anchored α-Amylase Activity……………………………………………………… 50

Figure 4.7: α-Amylase Activity in Intact NRRL Y-132/pAMY Measured by the DNS Assay…………………………………………………………… 52

Figure 4.8: α-Amylase Activity in Intact NRRL Y-132/pAMY Measured by the Iodine Assay…………………………………………………………..53

Figure 4.9: Batch Fermentation of NRRL Y-132/pAMY on Glucose……………………...56

Figure 4.10: Batch Fermentation of NRRL Y-132 and NRRL Y-132/pAMY on Soluble Starch ……………………………………………………………...57

Figure 4.11: Starch Hydrolysis in Batch Fermentation with Starch as
the Sole Carbon Source…………………………………………………….59
Figure 4.12: Batch Fermentation of NRRL Y-132 with Addition of
15 g/L of Barley α-Amylase………………………………………………..61
Figure 4.13: Batch Fermentation with the Addition of 1 g/L of Glucoamylase…………….62
Figure 4.14: Batch Fermentation with the Addition of 15 g/L of Barley α-Amylase
and 1 g/L of Glucoamylase……………………………………………... 64
Figure 4.15: Effects of SyntheticYeast Media on the Iodine Assay when Measuring
Starch Concentration……………………………………………………….66
Figure 4.16: Effect of Blasticidin Selection Pressure on Amylolytic Activity of
NRRL Y-132/pAMY Using the Starch Plate Assay…………………………70
Figure 4.17: Plasmid Stability During Batch Fermentation Using NRRL Y-132/pAMY
with the Addition of Amylolytic Enzymes…………………………………... 72
Figure 4.18: Plasmid Stability During Batch Fermentation Using NRRL Y-132/pAMY
without Addition of Amylolytic Enzymes…………………………………… 73
Figure 4.19: Structure of a Fusion Protein Containing Flocculation protein 1
as the Anchoring Protein………………………………………………….... 83

LIST OF ABBREVIATIONS

Ampicillin	Amp
Alcohol Dehydrogenase 1	ADH1
Blasticidin	Bsd
3, 5-Dinitrosalicylic Acid	DNS
Endoplasmic Reticulum	ER
Escherichia coli	*E. coli*
Ethylene-Diamine Tetraacetic Acid	EDTA
Glyceraldehyde 3-Phosphate Dehydrogenase	GAPDH
Glycosylphosphatidylinositol	GPI
Lithium Acetate	LiAc
Luria-Bertani	LB
Luria-Bertani Ampicillin	LBA
Multiple Cloning Site	MCS
Saccharomyces cerevisiae	*S. cerevisiae*
Sodium Dodecyl Sulfate	SDS
Terrific Broth	TB
Yeast extract, peptone and dextrose	YPD

1.0 INTRODUCTION

The baker's yeast, *S. cerevisiae*, is generally considered an efficient expression system for heterologous genes. Yeast has a eukaryotic cellular organization similar to those of plants and animals, making it a desirable host for the production of proteins that require posttranslational modifications for full biological activities. Functional amylolytic enzymes from various sources have been expressed and secreted at high levels in yeast (Rothstein *et al*., 1984; Filho *et al*., 1986; Toshihiko *et al*., 1986; Wong *et al*., 2002).

It has been suggested that using genetically engineered yeast expressing amylolytic enzymes could greatly advance bioethanol production at the industrial scale (de Moraes *et al*., 1995; Murai *et al*., 1997; Lipke *et al*., 1998; Murai *et al*. 1999; Kondo *et al*., 2002; Matsumoto *et al.*, 2002). However, genetic modification may cause changes in yeast metabolic pathways, and could affect yeast proliferation and ethanol production during fermentation. In addition, the use of yeast cell wall anchoring proteins, such as α-agglutinin, to immobilize active enzymes on cell surface has been intensively studied (Lipke *et al*., 1989, 1992; Cappellaro *et al*., 1994; Chen *et al*., 1995; Vaart *et al*., 1995; Schreuder *et al*., 1996; Vaart *et al*., 1997 Zou *et al*., 1995; Shen *et al*., 2001). This approach has been shown to have several advantages compared with secreted enzyme systems where amylolytic enzymes are expressed and secreted into the culture medium. However, cell surface anchoring may result in altering the protein native structure thereby leading to changes in enzymatic activity. Although many studies have been done on anchoring different starch hydrolysing enzymes on the yeast cell surface, the issues described above have not been fully addressed. Currently, most of the different methods for converting starch into ethanol by recombinant yeast are very complicated and not particularly efficient. The development of cold starch hydrolysis eliminated the cooking step in traditional starch hydrolysis process by liquefing and saccharifing raw starch directly with amylolytic enzymes at temperatures below the gelatinisation temperature of starch (Hill *et al*., 1997; Textor *et al*., 1998). Barley α-amylase was found to be superior to bacterial and fungal α-amylase for cold hydrolysis of wheat starch (Hill *et al*., 1997; Textor *et al*., 1998). This finding

suggested that a novel yeast strain could be designed to optimize the amylolytic activity and ethanol production of recombinant yeast in the fermentative process.

My objective was to develop a novel yeast strain which expressed and anchored barley α-amylase on the cell surface, and to test its ability to perform starch hydrolysis. Moreover, my goal was to examine the effects of over-expression of barley α-amylase on the recombinant yeast's ability to proliferate and produce ethanol under fermentation conditions.

The following literature review focuses on previous efforts that have been undertaken to generate yeast stains that express amylolytic enzyme, especially those that have employed a cell wall anchoring system.

2.0 LITERATURE REVIEW

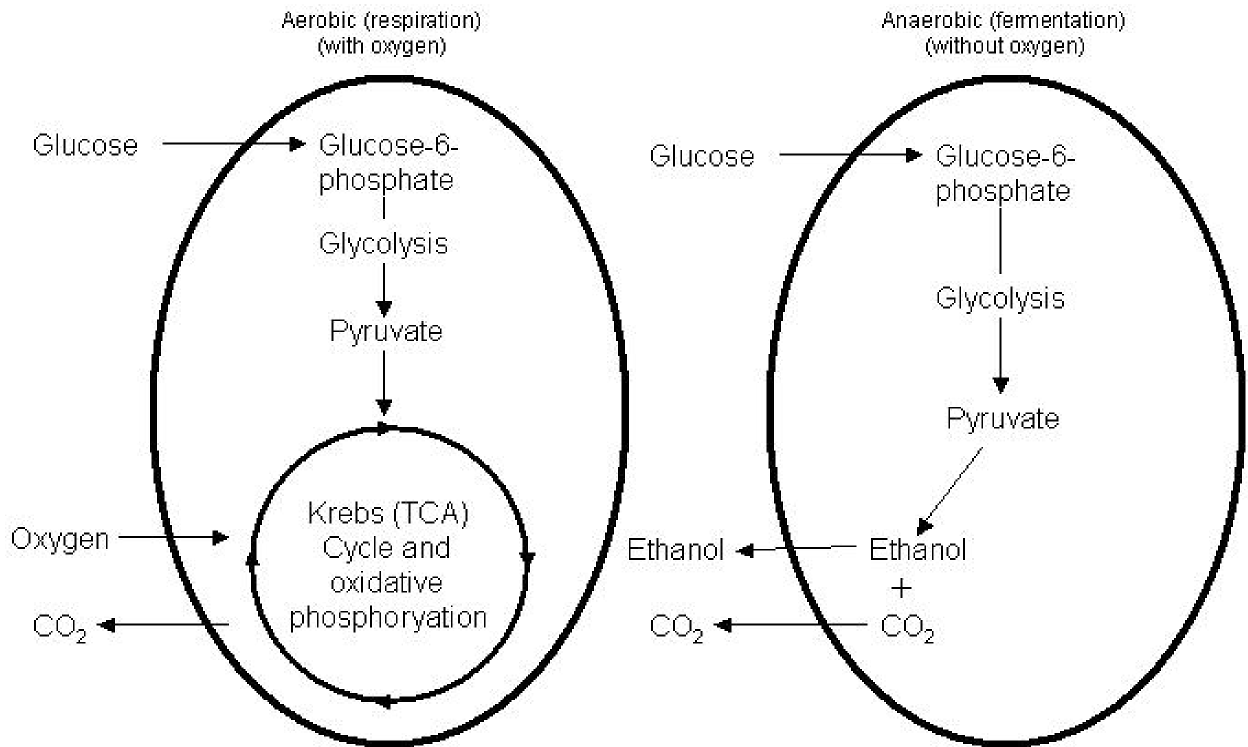
2.1 Bioethanol

The energy crisis has become a major issue due to the depletion of fossil fuels. The demand for an economical renewable energy resource is urgent. In comparison to many other fuel resources, ethanol has advantages of being renewable and environmentally friendly: it is biodegradable and burns cleanly with less generation of greenhouse gases than fossil fuels. Ethanol can contribute to the solution related to the diminishing supplies of fossil fuels and environmentally related problems that we are currently facing.

Bioethanol is referred to ethanol produced from the fermentation process performed by microorganisms. The brewing industry has been using this technique for decades to produce wines and beers. The baker's yeast, *S. cerevisiae* is the primary microorganism used for industrial bioethanol production. When incubated under anaerobic conditions, *S. cerevisiae* converts each glucose residue into two ethanol molecules and two carbon dioxide molecules with the net generation of two ATPs. The process is generally referred to as fermentation (Figure 2.1). Although sugar cane is reported to be the most widely used raw material for bioethanol production (Lang *et al*., 2001), in North America, starch is currently the most economical raw material (Ingledew, 1993; Lang *et al*., 2001; Bothast *et al*., 2005).

Starch is a product of our agriculture industry. In the USA, corn is the major source of starch; in Western Canada, starch is mainly supplied by wheat with 50% of it being produced in Saskatchewan (Lang *et al*., 2001; Bothast *et al*., 2005). Studies have suggested that the bioethanol industry could potentially benefit farmers and rural areas, and have positive effects on agricultural economics (Lang *et al*., 2001; Bothast *et al*., 2005; Editorials *Nature Biotechnology*, 2006). In addition, production of bioethanol from starch could reduce the consumption of fossil fuels and also reduce the net emission of CO_2 (Bourne, 2007).

However, there have been many concerns regarding the use of starch as the substrate for bioethanol production. The use of corn and wheat for bioethanol production has been causing a dramatic rise in food cost over the past 10 years (Bourne, 2007; Robert, 2007). Brazil has been successful at using sugar cane as a substrate for bioethanol production and this industry has



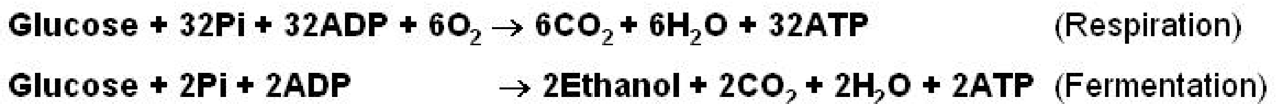


Figure 2.1 Aerobic and Anaerobic Catabolic Pathways for Glucose Utilization in Yeast. Yeast is able to utilize glucose aerobically (respiration) and anaerobically (fermentation). Glucose is converted into pyruvate via the glycolytic pathway, and then pyruvate is converted into carbon dioxide and energy (with oxygen) or is converted into ethanol, carbon dioxide and less energy (without oxygen).

been greatly reducing the country's reliability on fossil fuels (Bothast *et al*., 2005; Bourne, 2007; Robert, 2007). However, the climate of North America is not suitable for wide cultivation of sugar cane. In addition, studies have estimated that even if all of the crops available in North American were used for bioethanol production, the supplied amount of starchy materials would be far from sufficient if bioethanol is going to replace fossil fuel as the main energy source (Bourne, 2007; Robert, 2007; Wald, 2007). Further complications arise when determining if ethanol from starch is an environmentally friendly process. Some reports suggest that the use of large quantities of nitrogen fertilizer, pesticides and herbicides that is associated with excessive cultivation of corn and sugar cane can cause enormous contamination to surrounding environments and rivers (Editorial *Nature Biotechnology*, 2006). Furthermore, a study done by a research group at the University of California, Berkeley, reported that current bioethanol production based on corn fermentation could only reduce greenhouse gas emission by 18% compared to gasoline (Farrell *et al*., 2006). They also suggested that in order for ethanol production to contribute to energy and environmental goals in the future, starch will have to be replaced by cellulose as the substrate for fermentation processes.

Cellulose is a much cheaper carbon resource and available in abundance. However, it has a more complex structure than starch. Cellulosic materials that could be potentially used for bioethanol production can be obtained from agricultural residues such as leaves, stalks and husks of corn plants, forest wastes such as wood chips and tree barks, paper pulp and grasses (Farrell *et al*., 2006; Bourne, 2007; Robert, 2007; Wald, 2007). Studies have been suggesting that bioethanol produced from cellulosic materials could reduce greenhouse gas emission by 88%-90% compared to gasoline (Farrell *et al*., 2006; Bourne, 2007). Even though still in its early stage, much effort has gone into the investigation to commercialize cellulosic fermentation (Ho *et al*., 1999; Aristidou *et al*., 2000; Lynd *et al*., 2005; Himmel *et al*., 2007).

However, these issues do not reduce the importance of starch fermentation research. An editorial article "Bioethanol needs biotech now" in Nature Biotechnology (2006) stated that even though in the long-term bioethanol production from cellulose is the best way to contribute to our energy and environment goals, currently starch fermentation is the most economical way for bioethanol production, and biotechnology should play an important role in advancing starch fermentation into a more economical and environmentally friendly industry. Furthermore,

research discoveries on starch fermentation could also potentially benefit cellulose fermentation in the future.

2.2 Current Industrial Starch Fermentation Process

Starch exists as insoluble polymers of glucose residues linked to each other by α-1,4 and α-1,6 covalent bonds. Glucose residues linked by α-1,4 bonds form a linear chain. Single residues on the linear chains can also attach to other glucose molecules by a α-1,6 bond to form branched chains. Starch molecules ($(C_6H_{10}O_5)_n$) arranged solely by α-1,4 glucose linkages are linear, and are known as "amylose". When α-1,6 linkages are introduced into the amylose chain, the chain with branches is known as "amylopectin" (Figure 2.2). In normal corn starch, 27% is amylose and 73% is amylopectin (Bothast *et al.*, 2005).

Although starchy materials are available in abundance as carbon sources for cultivation, *S. cerevisiae*, the key organism used for alcohol fermentation, lacks amylolytic activity and is unable to directly utilize starch as a carbon source. Today, the process whereby most bioethanol is produced by fermentation can be generally categorized into two major steps. The first is starch hydrolysis. The raw starchy materials such as corn kernels are first crushed in a harmmermill and then slurried with water to form a mash. Next, with the addition of a thermostable α-amylase, the starch in the mash is gelatinized and broken down into dextrins by cooking the mash at high temperatures. This step is generally referred to as liquefaction. Following liquefaction, the dextrinized starch is cooled and further hydrolyzed into glucose residues by another starch hydrolysing enzyme glucoamylase. In the second step, after completion of starch hydrolysis, the mash that is composed mostly of glucose is mixed with yeast, which ferments the sugars into ethanol and carbon dioxide under anaerobic conditions. This step is generally referred to as fermentation. α-Amylase (EC 3.2.1.1, 1,4-α-D-glucan-4-glucanohydrolase) performs random endoamylolytic cleavage of 1,4-α-glycosidic linkages in starch to release oligosaccharides. Glucoamylase (EC 3.2.1.3, 1,4-α-D-glucanglucohydrolase) cleaves starch or oligosaccharides exoamylolytically, resulting in the removal of a single glucose residue one at a time from the reducing end of the polymer. Normally, α-amylase is first added to break down the long chains of the starch molecule into small pieces to generate more reducing ends for glucoamylase to attack. Glucoamylase will then digest these

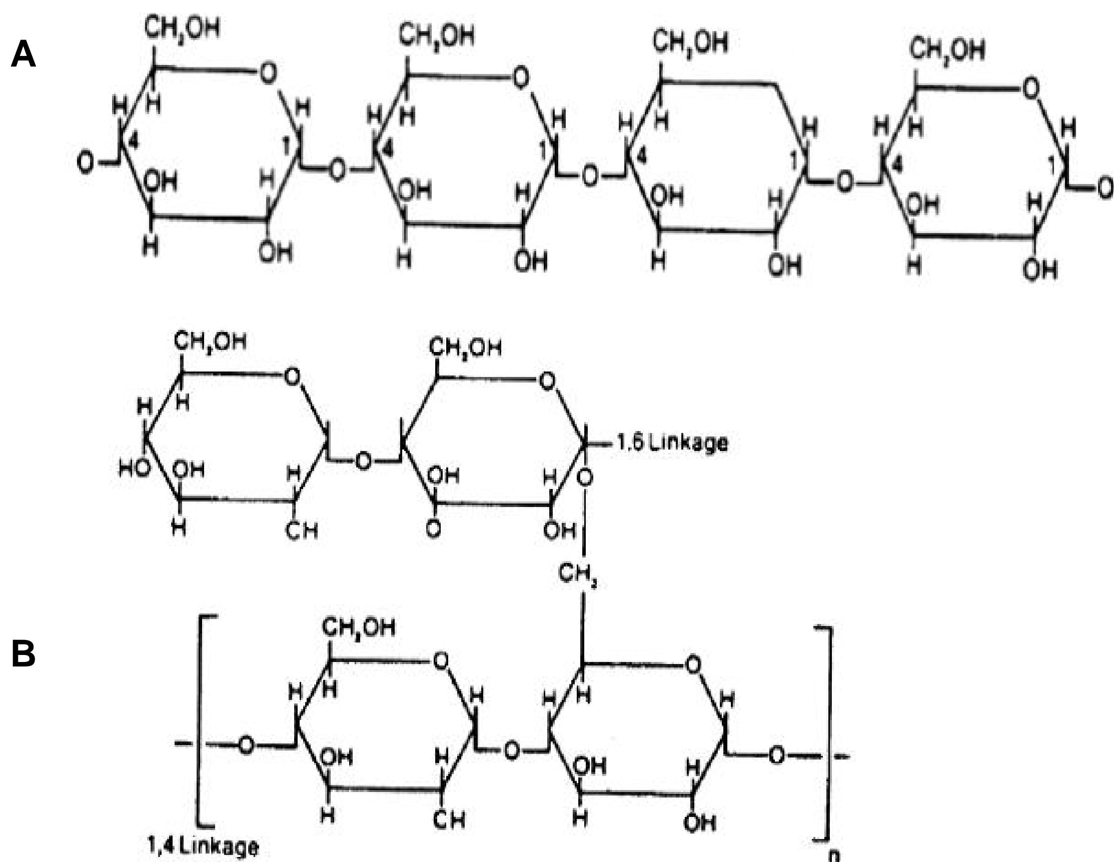


Figure 2.2 Structure of Amylose and Amylopectin. In amylose, the glucose residues are linked together by α-1,4 linkage (A). In amylopectin, parallel amylose chains are linked together by two glucose residues at the branch points by α-1,6 linkage (B). The diagram is taken from "Starch in general" by Archer Daniels Midland Company.

oligosaccharides into glucose. Both enzymes working together greatly increase the rate and efficiency of starch hydrolysis (Figure 2.3).

Although this process is commonly used today, and has been well studied to reduce the costs and give the maximum yield of ethanol, several disadvantages are limiting ethanol as a competitive fuel for the future. A large amount of energy is required during the pre-cooking process for gelatinizing the starch granules, consisting of 30–40% of all energy spent for ethanol production (Long *et al*., 2003). Additionally, a large amount of enzyme primarily α-amylase, is used in starch hydrolysis, and it was estimated that this costs contributes about 11% to the total annual operating expense for a cold starch hydrolysis plant (Lang *et al*., 2001). Also, special equipments are needed for the pre-cooking process, such as heat exchangers, steam jet cookers and holding tanks. Collectively, these factors greatly increase the eventual production costs of ethanol fermentation, and limit the economic potential of bioethanol as a fuel.

2.3 Application of DNA Recombinant Techniques to Make Bioethanol Production More Economically Competitive

Much effort has already been completed to reduce the capital cost of ethanol production. The development of cold starch hydrolysis has greatly reduced the thermal energy investment in the pre-cooking step (Textor *et al*., 1998; Lang *et al*., 2001). During the last 30 years, with the rapid development of DNA recombinant biotechnology, researchers have realized that construction of a genetically modified yeast strain with amylolytic activity, that can directly utilize starch for proliferation and fermentation, could greatly reduce the operation, equipment and enzyme costs on fuel bioethanol production.

2.3.1 Recombinant Yeast Strains that Secrete Starch Hydrolysing Enzymes

As stated in section 2.2, wild type *S. cerevisiae* strains are unable to directly utilize starch materials because they lack starch-hydrolysing activity. Two key starch-hydrolysing enzymes, glucoamylase and α-amylase, are generally used in bioethanol production to fully break down the starch polymers into single glucose residues. By using DNA recombinant technology, a gene coding for specific starch hydrolysing enzymes can be inserted into a designed DNA expression vector, called a plasmid.

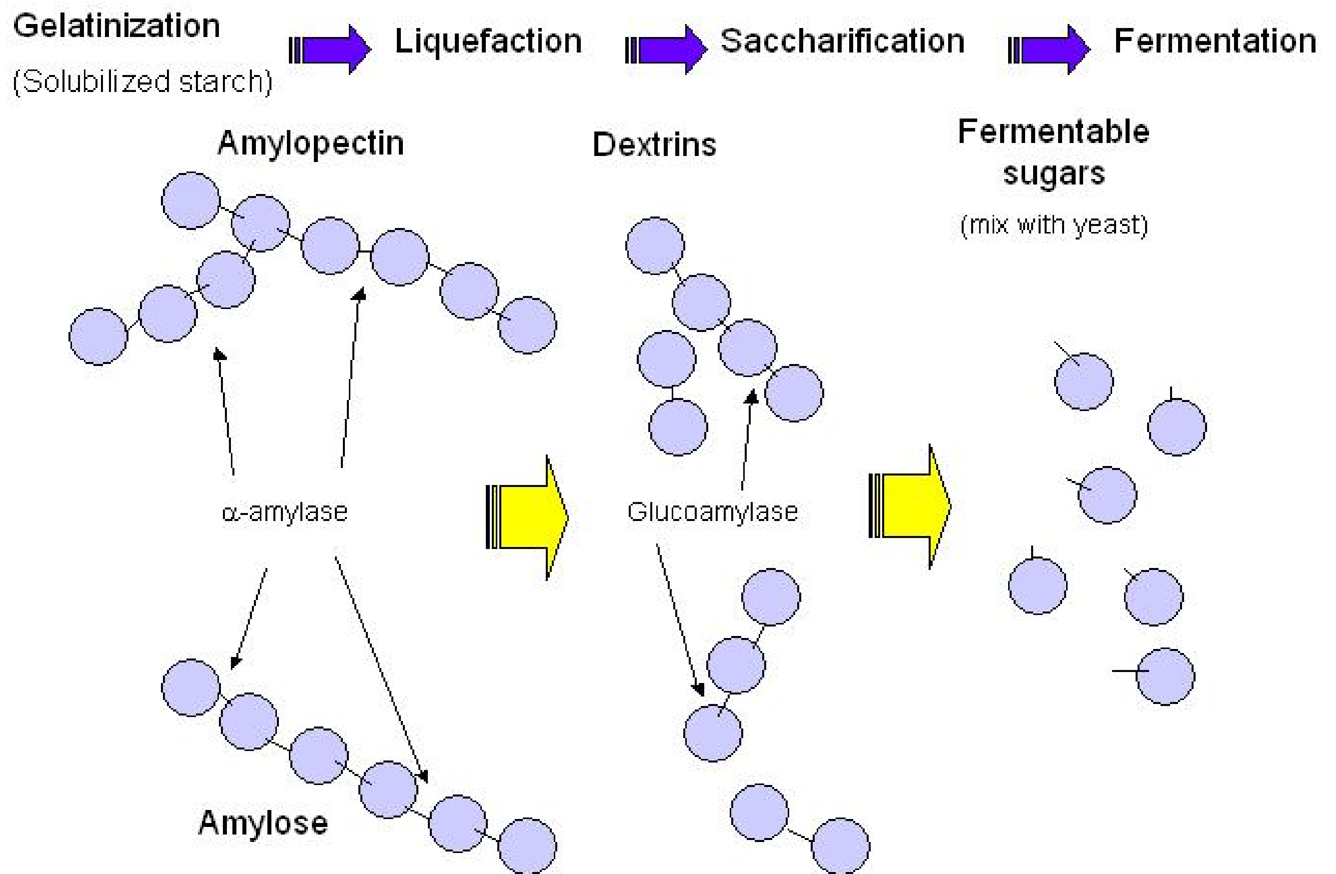


Figure 2.3 Schematic Representation of the Action of Amylases on Starch. Raw starch is slurried with water and then cooked at high temperature to its gelatinized state (more soluble), and with addition of thermal stable α-amylase, the mash is further liquefied into dextrins (referred to as liquefaction). Next, the mash is cooled and glucoamylase is added to convert the dextrins into fermentable sugars (referred to as saccharification). Then yeast is added to the mash to ferment the sugars to ethanol and carbon dioxide (fermentation).

After yeast are transformed with the plasmid, it is recognized by the cell's gene expression machinery, and the gene coding for the starch-hydrolysing enzyme is expressed. If the gene is attached to a secretion signal, the expressed enzyme can be exported out of the cell. Such genetically modified yeast, called recombinant yeast, should be able to utilize starch for proliferation and fermentation simultaneously.

Yeast is considered to be a good expression system for heterologous proteins (Schreuder *et al*., 1996). Many studies have demonstrated expression and secretion of functional starch hydrolysing enzymes from various sources by genetically modified yeast strains. Many strains were shown to be able to utilize soluble or raw starch as the sole carbon source for proliferation. Rothstein *et al*. (1984) genetically engineered a yeast strain secreting functional wheat α-amylase. The expression level was relatively low, and the secreted α-amylase showed reduced activity compared to that purified from wheat seeds. The ability of the recombinant yeast to grow on starch was not studied at that time. Toshihiko *et al*. (1986) genetically engineered *S. cerevisiae* XS-30-2B to express *Rhizopus oryzae* glucoamylase. This particular strain was able to grow on starch as the sole carbon source. However, they reported that the expression level of glucoamylase was not sufficient to be commercialized for starch fermentation, since high-level expression of amylolytic enzymes is important for achieving efficient starch hydrolysis and utilization. Following this work, by employing the *GAPDH* constitutive promoter, Toshihiko *et al*. (1989) were able to achieve high-level expression (above 300 mg/L) of *Rhizopus oryzae* glucoamylase in *S. cerevisiae* EH13-15. In addition, other studies were able to show that *GAPDH* and *ADH1* promoters are strong constitutive promoters, and both promoters were shown to provide high-level expression of heterologous proteins in yeast (Ruohonen *et al*., 1995; Vainio, 1994; Bitter *et al*., 1984).

Effective secretion of heterologous proteins was also considered to be a critical step. Research showed that the secretion signals of many precursor proteins from yeast, bacteria or mammals could be recognized by the yeast protein secretion machinery and used to direct efficient secretion of heterologous proteins from the yeast. Studies done on bacterial amylolytic enzymes showed that functional prokaryotic α-amylase from *Bacillus amyloliquefaciens* with its own secretion signal peptide (Ruohonen *et al*., 1987) or with a yeast pheromone α-factor secretion signal peptide (Southgate *et al*., 1993) could be expressed and secreted at high levels in yeast. Similar studies were carried out with mouse pancreatic α-

amylase, which was expressed and secreted at high levels by attaching to a yeast pheromone α-factor secretion signal sequence (Filho *et al*., 1986). In addition, barley α-amylase has been expressed and secreted by a genetically modified *S. cerevisiae* in a highly active form (Wong *et al*., 2002).

To further improve rate of the starch hydrolysis performed by amylolytic enzyme expressing recombinant yeasts, Steyn *et al*. (1991) were able to show that co-expression of glucoamylase and α-amylase was much more efficient for starch hydrolysis than if only one of the starch hydrolysing enzymes was expressed. However, interestingly, if both enzymes were expressed as a fusion protein, their activities were both reduced (Moraes *et al*., 1995).

These studies demonstrated that it is feasible to express and secrete highly active heterologous glucoamylase and α-amylase in yeast with high expression levels. In addition, related studies were able to show that some recombinant yeast strains that secrete amylolytic enzymes were able to utilize soluble starch for proliferation and fermentation (Nakamura *et al*., 1996, 2002; Birol *et al*., 1998). However, problems in these studies include in complete hydrolyses of starch during the fermentation process and ethanol production was relatively low. Deactivation of secreted amylolytic enzymes and loss of plasmids in recombinant yeasts through prolonged incubation are also important issues in recombinant yeast strains that secrete amylolytic enzymes.

2.3.2 Recombinant Yeast Strains With Cell Surface Anchored Amylolytic Enzymes

2.3.2.1 Enzyme Stability

As described above, enzyme stability can be a limiting factor for efficient starch hydrolysis. Also as demonstrated by some early studies, enzyme stability can affect the cost of ethanol production since high enzyme stability can reduce the cost for enzymes (Kvesitadze *et al*., 1982; Kondo *et al*., 1994; Leng *et al*., 2003). Enzymes secreted by recombinant yeast often become rapidly deactivated during fermentation (Kvesitadze *et al*., 1982; Kondo *et al*., 1994; Leng *et al*., 2003). Even though the enzymes can be continuously expressed and secreted into the fermentation broth, the accumulation of a large amount of deactivated enzymes could cause difficulty in ethanol distillation where ethanol is purified and concentrated. In addition, it is a

waste of fermentable sugars, which have to be continuously used for enzyme production rather than for the production of ethanol.

Studies have been carried out to physically attach enzymes to a solid surface to improve their thermal and operational stability. It was reported that the thermostability of barley α-amylase could be greatly enhanced by covalent immobilization on silica particles (Long *et al*., 2003). However, the operational stability of immobilized barley α-amylase did not change compared to soluble barley α-amylase under the same reaction conditions. This suggests that during starch hydrolysis, the immobilized enzymes tend to deactivate at the same rate as the soluble enzymes. In addition, product inhibition was not improved for immobilized barley α-amylase, suggesting that maltose and glucose still inhibited the immobilized enzymes in a similar manner as the soluble enzyme.

2.3.2.2 A Cell Wall Anchoring Protein α-Agglutinin and GPI Anchor

With accumulating studies on yeast cell wall structure and cell wall involved proteins, it was suggested that heterologous proteins could be immobilized on the yeast cell wall by being expressed as fusion proteins with one of the yeast's surface targeting proteins (Schreuder *et al*., 1996). Many different cell wall proteins have been identified, such as cell wall protein 1 (Cwp1), cell wall protein 2 (Cwp2), flocculation protein 1 (Flo1) and α-agglutinin (αGA1) (Vaart *et al*., 1995; Schreuder *et al*., 1996; Vaart *et al*., 1997). Among those cell wall target proteins, α-agglutinin is the one that has been studied the most (Lipke *et al*., 1989, 1992; Cappellaro *et al*., 1994; Chen *et al*., 1995; Zou *et al*., 1995; Shen *et al*., 2001; Zhao *et al*., 2001; Huang *et al*., 2003).

The cell wall anchoring mechanism of α-agglutinin involves many sub-cellular compartments in the yeast secretory pathway and many components in the cell wall. The cell wall of *S. cerevisiae* is mainly composed of glucan and mannoproteins. Glucan, which is composed of β-1,3 and β-1,6 linked glucose, is complexed with chitin to provide mechanical strength to the cell wall. β-1,3 glucan forms a fibrous network, but β-1,6 glucan is highly branched. The outer layer of the cell wall is coated with mannoproteins, which are highly glycosylated. Lipke *et al*. (1998) suggested that mannoproteins are covalently linked to glucan,

because they are resistant to extraction in hot SDS but can be cleaved from the wall by β-1,3- and β-1,6-glucanase (Figure 2.4).

α-Agglutinin was originally identified as a mannoprotein involved in the sexual adhesion of *S. cerevisiae* mating type α cells with *S. cerevisiae* mating type a cells (Lipke *et al*., 1989, 1992). α-Agglutinin consists of 650 amino acid residues, including a N-terminal secretion signal (1-19 residues), N-terminal binding domain (20-350 residues, consisting of three different domains that involve in interaction with a *S. cerevisiae* cells), a C-terminal Ser/Thr rich domain (351-627 residues) and a C-terminal GPI addition signal that is involved in cell wall anchorage (628-650 residues) (Chen *et al*., 1995). Evidence showed that α-agglutinin is covalently linked to the cell wall glucan (Lipke *et al*., 1989, 1992; Cappellaro *et al*., 1994). In addition, studies showed that the C-terminal Ser/Thr rich domain is extensively O-glycosylated, which suggests that this domain might have a rod-like conformation that acts as a spacer to extend the N-terminal binding domains to the cell surface (Lipke *et al*., 1989, 1992; Cappellaro *et al*., 1994; Chen *et al*., 1995; Zou *et al*., 1995; Shen *et al*., 2001) (Figure 2.5).

Schreuder *et al*. (1993) and Lu *et al*. (1995) showed that the 320 amino acids at the C-terminal half of α-agglutinin are responsible for the cell wall anchoring ability. A well-accepted explanation of the anchoring process of α-agglutinin is that the GPI anchor attachment signal is recognized in the yeast ER. Then, the hydrophobic attachment signal is replaced by a GPI anchor by a trans-peptide reaction in the ER. Following the attachment of the GPI anchor, α-agglutinin is transferred to the outer leaflet of the plasma membrane. The GPI anchor is then cleaved at its C-terminal glycan position and the remnant forms a glycosidic linkage with the branched β-1,6 glucan in the cell wall (Lipke *et al*., 1989; Lipke *et al*., 1992; Huang *et al*., 2003; Chen *et al*., 1995) (Figure 2.6).

2.3.2.3 Recombinant Yeast with Cell Surface Anchored Starch Hydrolysing Enzymes

As described above, by fusing starch-hydrolysing enzymes with α-agglutinin, the starch-hydrolysing enzyme can be stabilized on the yeast cell surface. The recombinant yeast strains with cell surface anchored starch hydrolysing enzymes have the advantage that the surface expressed enzymes are covalently linked to glucan in the cell wall, rendering them resistant to extraction. In 1997, Toshiyuki *et al*. (1997) anchored *Rhizopus oryzae* glucoamylase on the cell surface of *S. cerevisiae* MT8-1 by fusing the *Rhizopus oryzae*

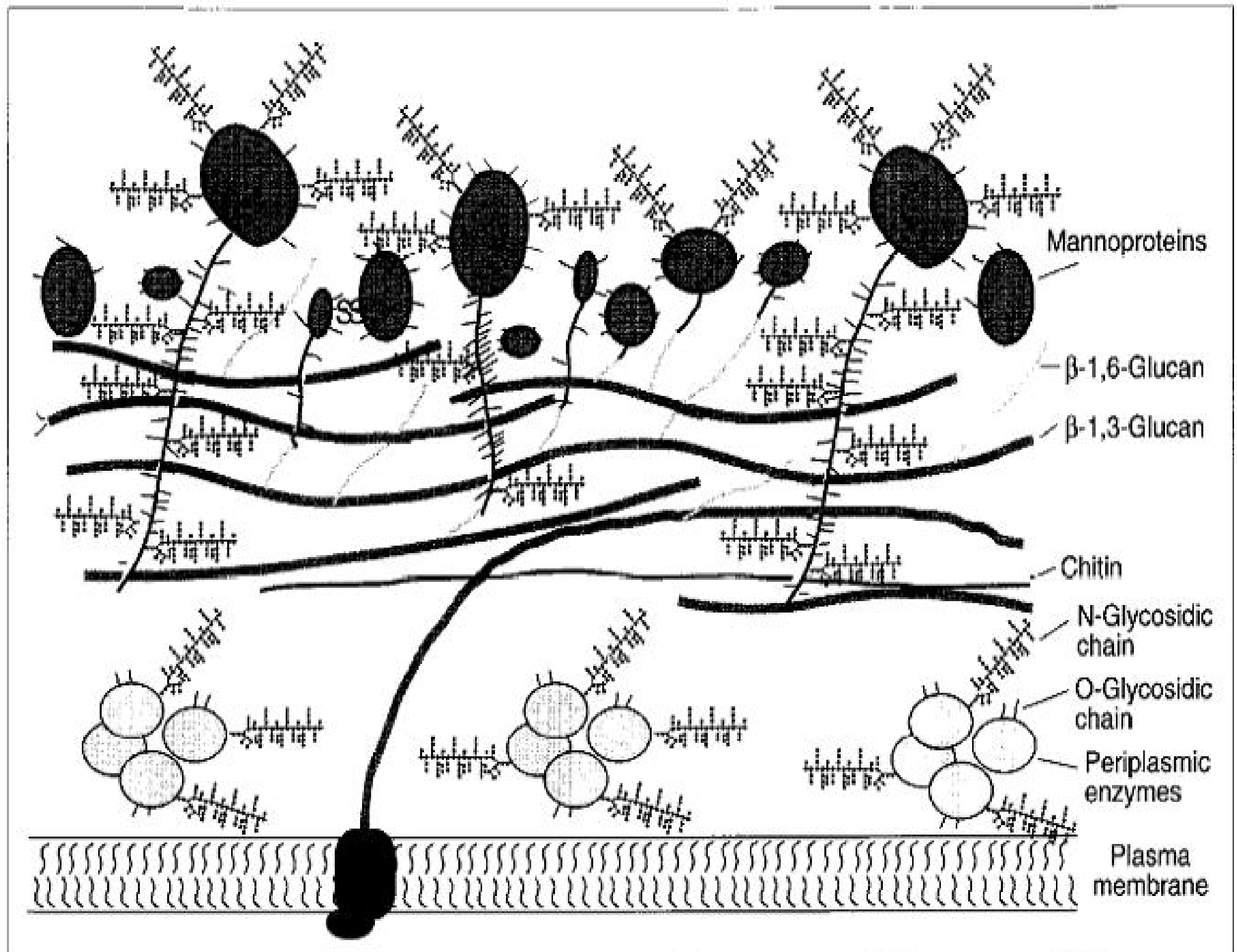


Figure 2.4 Structure of the Cell Wall of *S. cerevisiae*. The cell wall is located outside of the plasma membrane, and it is mainly composed of glucan and mannoproteins. The location of each component is shown above. The diagram is taken from Schreuder *et al.* (1996).

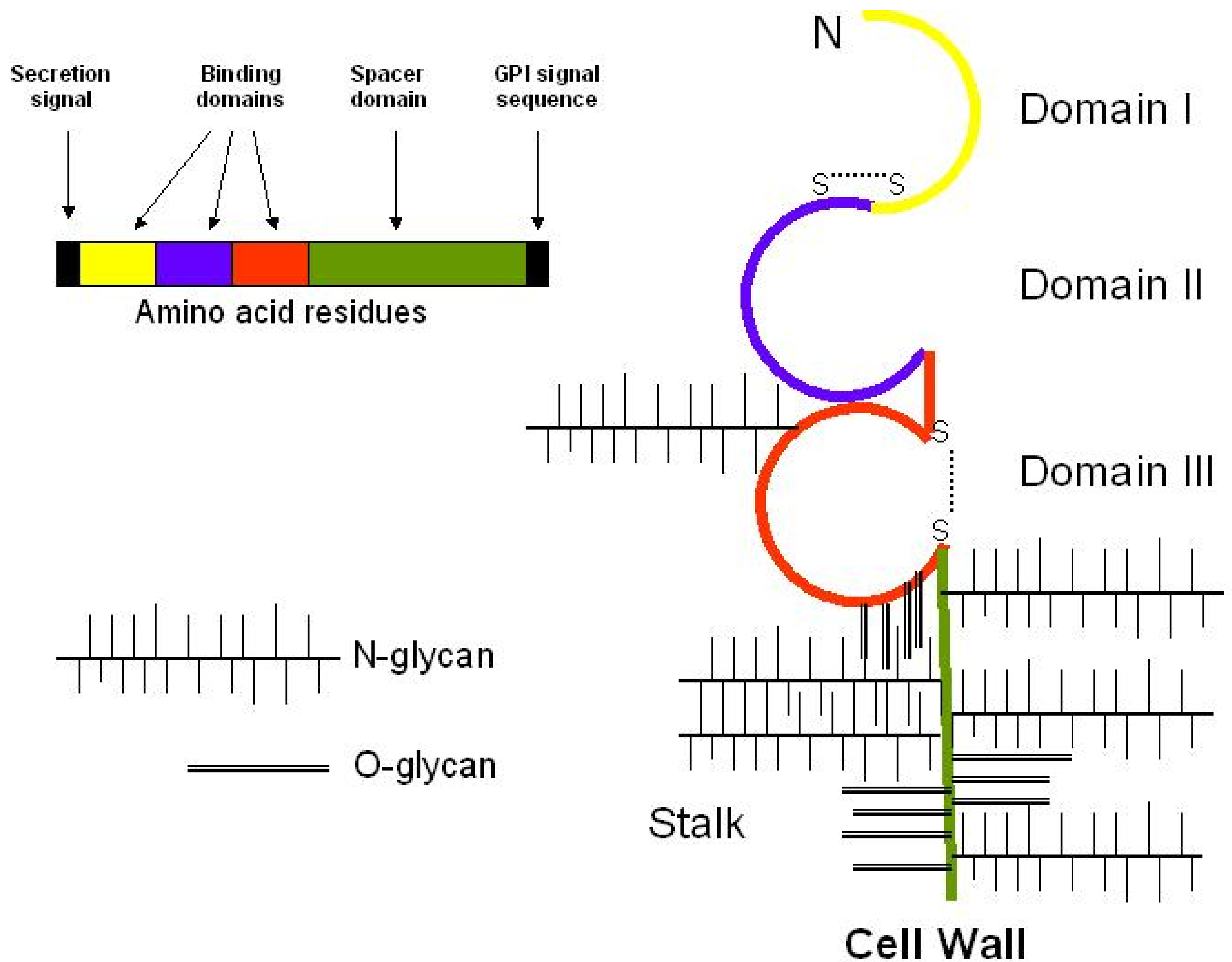


Figure 2.5 Structure of α-Agglutinin. The features of the α-agglutinin amino acid sequence are shown as indicated above (left panel). The structure of the cell wall anchored α-agglutinin is shown above (right panel) with the three binding domains at N-terminal and the spacer domain (stalk) spans the cell wall structure. The spacer domain is highly N-glycosylated and O-glycosylated as indicated. The diagram is taken from Chen *et al.* (1995).

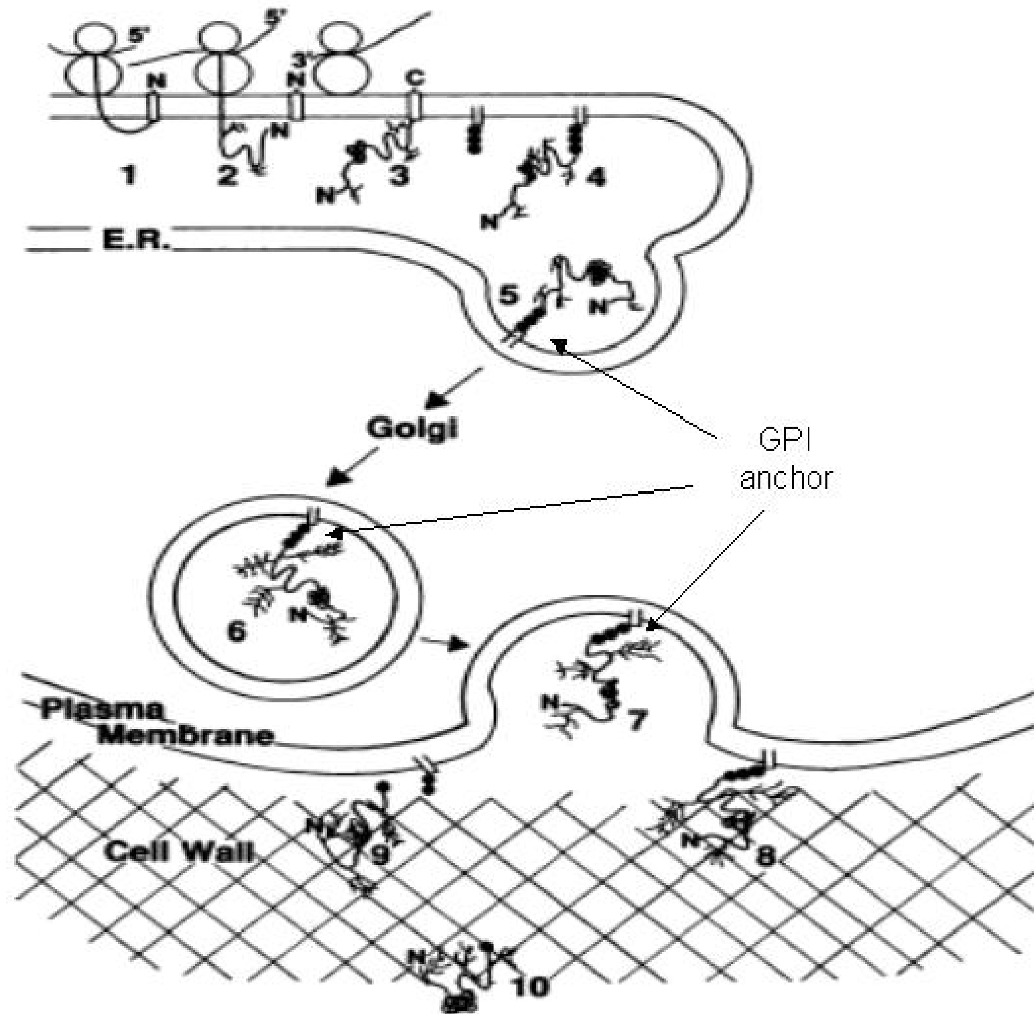


Figure 2.6 Schematic Representation of α-Agglutinin Localization by the GPI Anchor. In ER, the C-terminal hydrophobic sequence is cleaved, and a GPI anchor is attached (as the arrows indicate). Then the GPI attached α-agglutinin is transferred through Golgi and targeted to the cell membrane. After reaching the cell surface, α-agglutinin is cleaved from GPI anchor and transferred to β-1,6 glucan of the cell wall. Even though GPI anchor is still linked to the cell membrane, α-agglutinin is anchored in the cell wall structure at the final stage. The diagram is taken from Lipke *et al.* (1992).

glucoamylase cDNA with the 3' half of α-agglutinin cDNA, which is the part that has the GPI anchor attachment signal and is involved in anchoring proteins on the yeast cell wall. They also attached the secretion signal of the glucoamylase precursor protein at the 5' end of the fusion gene. This secretion signal sequence is a short peptide sequence that is recognized as an exporting signal in ER, such that the fusion protein attached to it is directed to the cell membrane. They hypothesized that when the fusion protein reaches the cell membrane, *Rhizopus oryzae* glucoamylase would be covalently linked to glucan in the cell wall through the GPI anchor, with the enzyme facing away from the cell wall structure. When the recombinant yeast is incubated with starch, the surface anchored glucoamylase would theoretically be able to break down starch and release glucose, which could in turn be utilized by the yeast for proliferation and fermentation. To test if the enzymes were covalently anchored on the cell wall, they first extracted the cell wall with hot SDS, which removed non-covalently bound proteins or proteins bound through disulfide bridges. Then, they treated the hot SDS extracted cell wall with β-1,3-glucanase. Their results showed that 93.2% of the total extractable glucoamylase was covalently anchored on the cell walls. The recombinant yeast cells were aerobically cultivated on 1% soluble starch as the sole carbon source for over 100 hours. They were able to show that the recombinant yeast could grow on starch and that cell growth was comparable to those grown on 1% glucose. The activity of cell wall anchored glucoamylase was comparable to secreted enzymes.

Murai *et al.* (1999) developed several recombinant yeast strains that displayed *Rhizopus oryzae* glucoamylase and/or α-amylase from *Bacillus stearothermophilus* on the surface of the cell wall. The enzymes were anchored with α-agglutinin. They found that the recombinant strains displaying either *Rhizopus oryzae* glucoamylase alone or both *Rhizopus oryzae* glucoamylase and *Bacillus stearothermophilus* α-amylase were able to grow on starch as the sole carbon source. In contrast, the recombinant strain displaying *Bacillus stearothermophilus* α-amylase alone was unable to grow on starch. Their results indicated a dramatic activity reduction for cell surface anchored *Bacillus stearothermophilus* α-amylase.

Kondo *et al.* (2002) genetically engineered a flocculant yeast strain *S. cerevisiae* YF207 by anchoring *Rhizopus oryzae* glucoamylase on the cell surface. They were able to achieve a high-level of ethanol production of 50 g/L on soluble starch. The average percentage of theoretical yield of ethanol was 100% based on seven, 120-hour batch fermentation runs using

this recombinant yeast strain. However, the starch degradation rate was relatively low compared to that using soluble enzyme. In order to enhance the strain's starch degradation rate and ethanol production rate, in a later study, *Rhizopus oryzae* glucoamylase and *Bacillus stearothermophilus* α-amylase were co-anchored on the yeast cell surface (Shigechi *et al*., 2004). This new recombinant yeast strain was able to directly utilize raw starch for fermentation. Shigechi *et al*. (2004) reported that in a 72-hour fermentation run, ethanol production from raw starch was 61.8 g/L with a theoretical yield of 86.5%. Two different anchoring proteins, α-agglutinin and flocculation protein 1, were utilized to attach *Bacillus stearothermophilus* α-amylase in the cell wall. α-Amylase anchored with α-agglutinin showed little activity; however, when anchored with flocculation protein 1, activity was restored. Shigechi *et al*. (2004) proposed that since the C-terminal of *Bacillus stearothermophilus* α-amylase is involved in starch binding, that when the C-terminal of the enzyme was fused to α-agglutinin, the anchoring might have hindered the enzyme's accessibility to its substrate. In contrast, flocculation protein 1, which is a cell wall protein involved in flocculation activity between yeast cells (Miki *et al*., 1982; Straveret *et al.*, 1994; Bony *et al.*, 1997), when attached to the N-terminal of *Bacillus stearothermophilus* α-amylase, allowed the enzyme's C-terminus to extend freely into the medium. Different from α-agglutinin, the N-terminal of flocculation protein 1 anchors itself on the cell wall by non-covalently attaching to the mannoproteins of the outer layer of the cell wall (Takeshi *et al*., 2002).

A similar study done by Takeshi *et al*. (2002) showed that *Rhizopus oryzae* lipase, which also has a C-terminal located substrate-binding site and showed no detectable activity when anchored in the cell wall with α-agglutinin, had its activity restored when anchored with Flo1. However, since enzymes anchored by Flo1 are not covalently linked to the cell wall structures, the anchored proteins could be extracted with hot SDS.

2.3.2.4 Domain Functions and Structure of Barley α-Amylase

Barley α-amylase has been intensively studied over the past 20 years since its high rate of hydrolysis towards raw starch has huge potential for industrial applications. Previous studies showed that in germinating barley seeds, there are different α-amylase isozymes, encoded by two multi-gene families and referred to as AMY1 and AMY2. The two isozymes were reported to display 80% sequence identity, with AMY1 composed of 414 amino acids and AMY2

composed of 403 amino acid residues. They are also distinguished by their pIs with AMY1 known as the low-pI (pI = 4.9) isozyme, and AMY2 as the high-pI (pI = 5.9) isozyme (Rogers *et al*., 1983, 1984; Rogers, 1985; Khursheed *et al*., 1988). Interestingly, studies showed that several features of the two isozymes differ greatly. Compared to AMY2, AMY1 is more stable at acidic pH, but less stable at elevated temperature (Rodenburg *et al*., 1994). Importantly, AMY1 was shown to have higher affinity and activity toward starch granules than AMY2 (MacGregor *et al*., 1980, 1986; Sogaard *et al*., 1990). These properties have made AMY1 more favourable for industrial application of starch hydrolysis.

A much more complete picture of the three-dimensional structure of AMY1 has been recently solved and the functions of the major domains were described (Kadziola *et al*., 1998; Gottschalk *et al*., 2001; Robert *et al*., 2003, 2005). The three dimensional structure of barley α-amylase is shown in Figure 2.7. Barley α-amylase is composed of three domains, including a major domain consisting of 288 amino acids forming a $(\beta/\alpha)_8$-barrel (domain A), a small loop with 65 amino acids protruding between β_3 and α_3 of domain A (domain B) and a C-terminal domain with 61 amino acids organized into five-stranded anti-parallel β-sheets (domain C). Domain A was first characterized and recognized as the main location of starch binding and catalytic activity. Recently, an additional starch-binding site was found in domain A. Its function was proposed to be involving in enhancing starch binding of the enzyme. Domain B is responsible for binding of calcium ions and maintenance of the enzyme's three-dimensional structure. Domain C has recently been recognized as the third potential starch-binding site in the enzyme. Robert *et al*. (2003) showed that even though AMY1 and AMY2 share high similarity in domain C, this binding site does not exist in AMY2. It has been suggested that the two extra starch binding sites located at domain A and C play important roles for orienting the substrates in the right position towards the enzyme's catalytic site, and are critical to barley α-amylase's starch hydrolysis rate (Robert *et al*., 2003, 2005). However, their specific role during the starch hydrolysis reaction needs to be further investigated.

2.3.3 Plasmid Stability

Plasmid stability in recombinant yeast can be an issue during prolonged fermentations when antibiotic selection is used. Plasmid stability is normally measured as a ratio of the

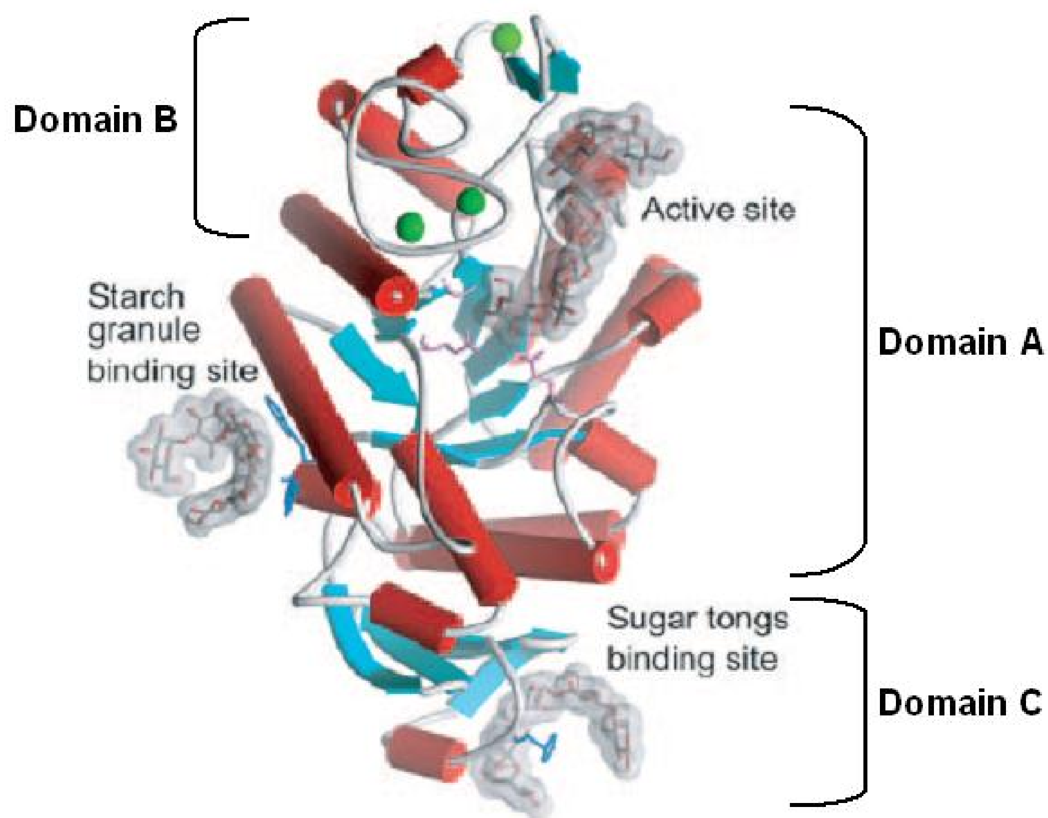


Figure 2.7 Three Dimensional Structure of Barley α-Amylase Isozyme AMY1. Calcium ions in domain B (green dots), the active site in domain A (top), the starch granule binding site in domain A (left) and the sugar tongs binding site (bottom) in domain C are shown as indicated above. The three binding maltoheptaose molecules (mimic molecules of short starch chain) are shown in a gray transparent surface. The diagram is taken from Robert *et al.* (2005).

number of plasmid bearing cells over that of the plasmid free cells at a certain time point during the fermentation (Filho *et al.*, 1986; Ruohonen *et al.*, 1987; Kondo *et al.*, 2002). Since the selection pressure is lost as a result of the degradation of the antibiotic, the plasmid-free cells grow whenever free fermentable sugars are available in the fermentor. The plasmid-free cells always overgrow the plasmid bearing cells under the conditions without enough selection pressures (Altintas *et al*., 2001; Nakamura *et al*., 2002). High plasmid stability is desired, since a greater plasmid stability corresponds to a higher ratio of the number of plasmid bearing cells over the number of plasmid free cells in the fermentor at the checked time point. Continuous addition of large amount of antibiotics can maintain high plasmid stability during fermentation; however, it is not applicable for industry ethanol production. The added antibiotics increase ethanol production cost and additional procedures have to be carried out to separate the antibiotics from the final products.

In some cases, instead of using antibiotics, plasmid stability of the recombinant yeast strains can be maintained by using minimal medium. Recombinant yeast strains selected in this way have to be yeast mutants with a genetic deficiency, and they are selected by transforming them with plasmids containing auxotrophic selection markers such as *Leu*$^-$ or *Trp*$^-$ (Murai *et al.*, 1997; Kondo *et al*., 2002). However, most industrial ethanol producing strains are not genetically modified in this way, and cannot be selected by using minimal medium.

It was reported that without selection pressures, plasmid-free cells can quickly overgrow plasmid-bearing cells (Alintas *et al*., 2001). Plasmid stability was studied based on data from starch fermentation using recombinant *S. cerevisiae* strain YPB-G, which expresses and secretes *Bacillus subtilis* α-amylase and *Aspergillus awamori* glucoamylase as a fusion protein. The recombinant yeast were pre-cultured in minimal medium, and used to inoculate a 2.5-litre bioreactor containing 30 g/L soluble starch at 30°C. A mathematical model was used to simulate plasmid stability in the fermentation broth and then actual experimental data was obtained to confirm the prediction from the model. Based on experimental observations, during the first generation, plasmid-free cells and plasmid-bearing cells grew at the same rate. However, starting in the second generation, the growth rate of plasmid-free cells surpassed plasmid-bearing cells and reached twice the growth rate of plasmid-bearing cells. Their results indicated that as more fermentable sugars became available, plasmid-free cells tend to outgrow plasmid-bearing cells, with the biomass of plasmid-free cells and plasmid-bearing cells

becoming 2.27g/L to 0.51 g/L, respectively. Based on the data, the authors suggested that high copy number plasmid is relatively stable under selection conditions but not suitable for large-scale fermentation in long term culturing conditions. They further suggested using an integrative plasmid to insert the DNA sequence into the yeast chromosome to solve the plasmid stability problem.

The results from the above study of Alintas *et al*. (2001) were based on recombinant yeast that were secreting starch-hydrolysing enzymes. However, the situation might be quite different if the enzymes are anchored on the cell surface. A recombinant yeast strain was constructed by introducing a multicopy plasmid containing a *Rhyzopus oryzae* glucoamylase/α-agglutinin fusion gene into a flocculent yeast strain YF207 (Kondo *et al*., 2002). The recombinant yeast was pre-cultured in minimal medium to ensure 100% plasmid-bearing cells. These pre-cultured yeast cells were used to inoculate a two litre bioreactor containing 40g/L of soluble starch at 30°C. Plasmid stability was monitored, and high plasmid stability was maintained during the fermentation (approximately 85-90%). The result indicates that approximately 90% of cells in the fermentation culture were plasmid-bearing cells. Interestingly, they were unable to detect glucose in the medium during the fermentation. Kondo *et al*. (2002) proposed that since the glucoamylase was anchored on the yeast cell wall, only those starch molecules close to the yeast cells were degraded by the enzymes, and the released glucose was readily taken up by these yeast cells. This suggests that there would be no accumulation of large quantities of glucose in the fermentation medium over time, consistent with their observations of glucose concentrations in the fermentor. If no free glucose was available in the medium, plasmid-free cells would not be able to proliferate. Thus, starch degradation ability potentially plays the role of a selection agent. By using the recombinant yeast with cell surface anchored glucoamylase during starch fermentation, contamination caused by bacteria would be prevented, since no free glucose would accumulate in the fermentation medium.

Based on the two studies with cell surface anchored starch-hydrolysing enzymes (Alintas *et al*., 2001; Kondo *et al*., 2002), recombinant yeast harbouring multiple-copy plasmids appear to work more efficiently for starch hydrolysis than yeast with integrated expression plasmids, as multiple-copy plasmids give 10 to 50-fold higher expression levels.

2.3.4 Cell Surface Anchored Yeast Expression System vs. Secreted Yeast Expression System

There are several disadvantages to using recombinant yeast strains that secrete amylolytic enzymes. Direct inoculation of recombinant yeast that secrete amylolytic enzymes into media that contains starch as the sole carbon source is impossible, since expression and secretion of amylolytic enzyme by the recombinant yeast is a prerequisite for starch utilization. Pre-cultivation is needed since recombinant yeasts need to express enough amylolytic enzymes to break down starch. In industry, yeast cells are normally recycled after each fermentation run, and in this case, the recycled yeast can not be directly used for the next fermentation process since they lack amylolytic activity. Yeast cells can be easily separated from the fermentation broth by gravity or centrifugation. Secreted amylolytic enzymes would be considered as contaminants during the fermentation process and would have to be separated from the ethanol product. This could increase costs in the distillation step. In addition, secreted amylolytic enzymes are quickly deactivated during fermentation, as they suffer the same thermal stability problems as added enzymes.

As mentioned in section 2.3.3, cell surface anchored yeast expression systems are more efficient for starch utilization than secreted expression systems. Cell surface anchored systems also have the advantage of being recycled with the cells after each batch fermentation. This is because all the enzymes are covalently linked to the cell wall, thus enzymes are easily recycled with cells, and no additional steps have to be taken to separate the enzymes from the final products. Recombinant yeast cells can be readily used for the next fermentation cycles, and no pre-culturing step is needed to express the amylolytic activity of the recombinant cells before they can perform starch hydrolysis.

2.4 Assays Used to Detect and Quantify Amylolytic Activities

2.4.1 Starch Plate Assay

The starch plate assay is widely used to screen for recombinant yeast that have amylolytic activities. The basic idea of this assay is to use iodine vapour to stain YPD/Starch

plates with transformed yeast colonies growing on it. Detection of α-amylase activity on the cell surface is based on halo formation around the colonies after staining with iodine vapour. However, it has been reported that this assay is not for quantitative purposes, since the size of the halo formation may not be proportional to the expression level and activity of amylolytic enzymes expressed by the recombinant yeast (Moraes *et al.*, 1995).

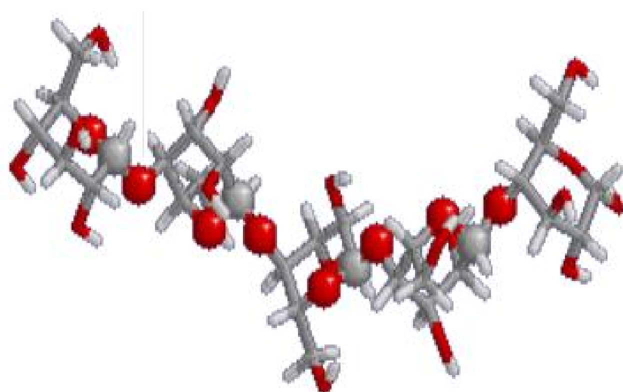
2.4.2 Iodine Assay

Amylose forms α-helical coils in three-dimensional space. In liquid solution, iodine molecules and iodide ions are able to fit into and line up inside the α-helical coils of amylose. The starch-iodine complex is soluble in water and gives a dark-blue colour. Other polysaccharides and monosaccharides cannot complex with iodine or iodide ions since they do not have similar α-helical structures (Figure 2.8). This feature is commonly used for measuring starch concentration and assaying for amylolytic activities (Filho *et al.*, 1986; Ruohonen *et al.*, 1987; Kondo *et al.*, 2002). The presence of amylolytic activity will cleave amylose into polysaccharides or monosaccharides, which can no longer complex with iodine or iodide ions. The amylolytic activity in the solution can be measured based on the reduction of the dark-blue colour in the assay.

The assay can only be performed on soluble starch and the colour reagent is commonly composed of iodine (I_2) and potassium iodide (KI) solutions. However, the starch-iodine complex is sensitive to elevated temperature and pH. Normally, the assay is performed at room temperature and at a pH lower than 8. Related studies have shown that the assay is sensitive to most bacteria and yeast growth media (Manonmani *et al.*, 1999).

2.4.3 3, 5-Dinitrosalicylic Acid (DNS) Assay

This assay is based on the detection of the presence of free carbonyl groups (C=O) in a reaction solution. Since reducing sugars, such as glucose, fructose, lactose and maltose, contain a free carbonyl group (C=O) at their reducing end, the assay is commonly used to detect and quantify amylolytic activity which generates reducing sugars from starch polymers. Using glucose as an example, the mechanism of the assay is shown in Figure 2.9. In liquid solution, glucose exists in equilibrium between its ring conformation and chain conformation. Under the DNS assay conditions, the aldehyde group from glucose is oxidized into a carboxyl group, and



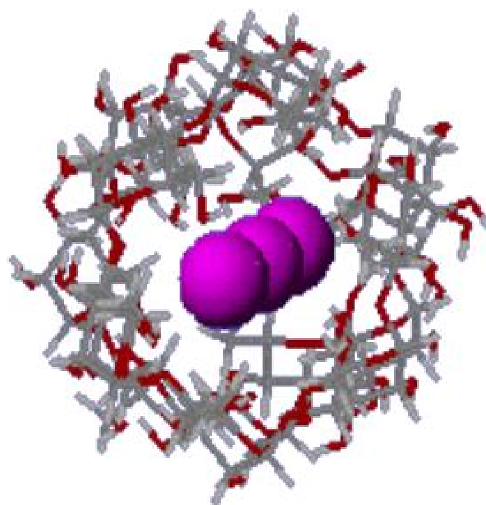


Figure 2.8 Structure of the Starch-Iodine Complex. The side view of the α-helical coils formed by amylose in three-dimensional space (left panel). The front view of the starch-iodine complex (right panel). The iodine molecules and iodine ions (indicated as purple spheres in right panel) are able to fit into and line up inside the α-helical coils of amylose and the complex gives a dark-blue colour in solution. The diagram is taken from Ophardt, *Virtual chembook* (2003).

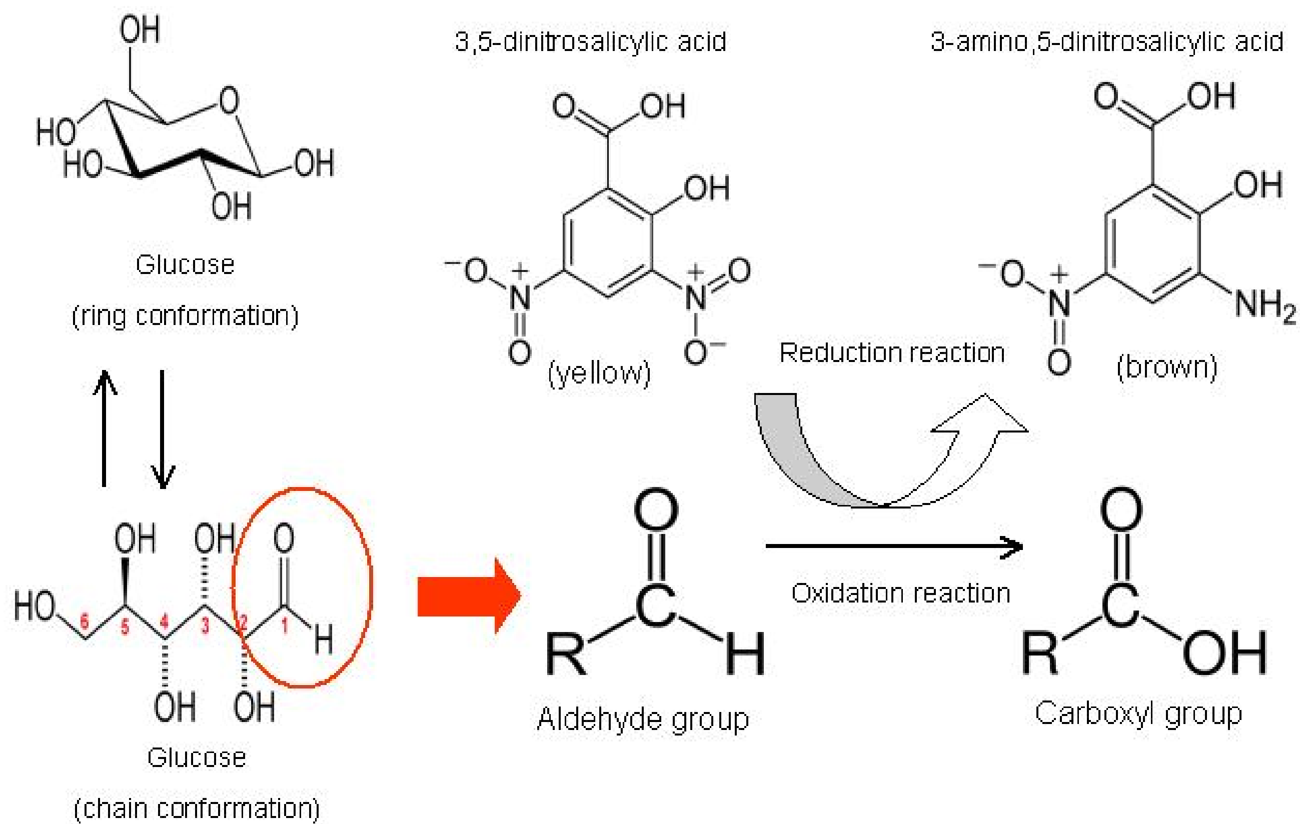


Figure 2.9 Reaction Mechanism of DNS Assay. In liquid solution, glucose exists in equilibrium between its ring conformation and chain conformation. Under the DNS assay conditions, the aldehyde group from glucose is oxidized to a carboxyl group, and at the same time, 3, 5-dinitrosalicylic acid is reduced into 3-amino, 5-nitrosalicylic acid.

at the same time, 3, 5-dinitrosalicylic acid is reduced to 3-amino, 5-nitrosalicylic acid. The reaction is accompanied by a colour change from yellow (3, 5-dinitrosalicylic acid containing solution) to brown (3-amino, 5-nitrosalicylic acid containing solution). Normally, the assay solution is boiled for 5-15 min to stabilize the colour formation. The wavelength of 540 nm was reported to give maximum absorbance for 3-amino, 5-nitrosalicylic acid (Miller, 1959).

2.5 Objectives

Currently, most of the methods for converting starch into ethanol by recombinant yeast are not particularly efficient. Barley α-amylase (α-1, 4 glucan glucanohydrolase) was found to be superior to bacterial and fungal α-amylase for cold hydrolysis of wheat starch at 45 °C and pH 4.5 by Textor *et al*. (2001). This suggested that a novel yeast strain could be designed to optimize amylolytic activity and ethanol production of recombinant yeast in a fermentation process. The goal of this project was therefore to engineer a novel genetically modified yeast strain expressing anchored barley α-amylase in the cell wall, and to study its ability to carry out hydrolysis and fermentation of soluble starch.

The specific objectives of this thesis were:

1. Construction of a novel plasmid capable of expressing and anchoring barley α-amylase on the yeast cell wall.
2. Transformation of yeast with the constructed plasmid and selection of yeast clones.
3. Detection and quantification of cell wall anchored α-amylase activity.
4. Examination of the ability of the recombinant yeast to proliferate and carry out fermentation on soluble starch.

3.0 MATERIALS AND METHODS

3.1 Reagents

The names of the reagents and their suppliers are listed in Table 3.1. The addresses of the individual suppliers are given in Table 3.4.

Table 3.1: Lists of Reagents and Suppliers.

General Reagent	Supplier
Absolute Ethanol	BDH
Agarose	Bio-Rad
Calcium Chloride	BDH
Chloroform	BDH
Dimethylsulfoxide (DMSO)	BDH
Ethidium Bromide	Sigma-Aldrich
Ethylene-Diamine Tetraacetic Acid Disodium Salt (EDTA)	BDH
Glacial Acetic Acid	EMD
D-Glucose	BDH
Glycerol	BDH
Hydrochloric Acid (HCl)	BDH
Isopropanol	BDH
Phenol	Sigma-Aldrich
Potassium Chloride	BDH

Sodium Acetate	BDH
Sodium Chloride	BDH
Sodium Hydroxide (NaOH)	BDH
Cell Culture Reagent	**Supplier**
Ampicillin	ICN
Blasticidin	Invitrogen
Bacto-Agar	DIFCO
Bacto-Tryptone	DIFCO
Bacto-Yeast Extract	DIFCO
Peptone	DIFCO

Table 3.2: List of Commercial Kits.

Commercial Kit	Supplier
Qiagen PCR Purification Kit	Qiagen
DNS Assay Kit	Sigma-Aldrich

Table 3.3: List of Oligonucleotides (Invitrogen) for PCR.

PF=Forward Primer PR=Reverse Primer

Primer Names	Primer Sequence
Primer I (PF)	5’–CGCCATCGATAAGAACGGCAG–3’
Primer II (PR)	5’–TTCAGCTCCGCTCGAGTGTTG–3’

Table 3.4: Names and Addresses of Suppliers.

Supplier	Address
BDH	501-45th Street West, Saskatoon, SK., Canada
Bio-Rad	5671 McAdam Road, Mississauga, Ont., Canada
DIFCO	7 Loveton Circle, Sparks, MI 48232-7058, USA
EMD Biosciences, Inc.	10394 Pacific Center Court, San Diego, CA 92121, USA
ICN	12 Morgan, Irvine, CA 92618-2005, USA
Invitrogen	100 Faraday Avenue, Carlsbad, CA 92008, USA
Sigma-Aldrich	2149 Winston Park Drive, Oakville, Ont., Canada
Qiagen	2800 Argentia Road, Unit 7, Mississauga, Ont. Canada

3.2 Bacteria Strain, Yeast Strains and Media Preparations

E.coli NM522 (New England, Biolabs) was used as the host for the propagation of plasmids.

Yeast strain *S. cerevisiae* NRRL Y-132 was used as the host to express cell wall anchored barley α-amylase (*S. cerevisiae* NRRL Y-132 is an ethanol producing yeast strain used in industrial bioethanol production). pAMY harbouring *S. cerevisiae* NRRL Y-132 was used in amylolytic assays and batch fermentation studies.

Two different media, LB and TB were used to cultivate and propagate *E. coli* NM522. LBA plates were used to cultivate and select transformed bacterial cells. LB consists of 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) sodium chloride. TB consists of 1.2% (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast extract, and 0.4% (v/v) glycerol. Both solutions were autoclaved for 20 min at 15 lb/sq. in. before used. TB was completed by adding 10% (v/v) of sterile 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 to the cooled medium after autoclaving. The solutions were placed at 4°C for long-term storage. LBA consists of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride and 1.5% (w/v) agar. After autoclaving, ampicillin was added to a final concentration of 100 μg/mL into the cooled medium. The medium was then mixed and poured into petri dishes. The LBA plates were placed at 4°C for long-term storage.

Two different media were used for yeast cultivation and fermentation. YPD medium consists of 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) D-glucose. This medium was used primarily for yeast pre-cultivation and propagation during competent yeast preparation and whole cell assays. For batch fermentation, yeast synthetic medium was used. It contains (w/v) 0.25% NH_4Cl, 0.291% Na_2HPO_4, 0.3% KH_2PO_4, 0.025% $MgSO_4$, 0.008% $CaCl_2$, 0.53% citric acid, 0.25% sodium citrate and 0.3% yeast extract. Both media were autoclaved before use. For batch fermentation, 2% (w/v) D-glucose or 2% (w/v) soluble wheat starch was added along with yeast cells into yeast synthetic media at the time of inoculation.

3.3 DNA Sub-cloning and Plasmid Construction

Protocols in this section are based on those described in Sambrook *et al.* (1989).

3.3.1 Polymerase Chain Reaction (PCR)

Two restriction digestion sites, *ClaI* (5' end) and *XhoI* (3' end), were generated at the ends of barley α-amylase (α-1, 4 glucan glucanohydrolase, EC 3.2.1.1, type VIII-A) cDNA by PCR (Table 3.3).

The PCR mixture was set up as follows (in a total reaction volume of 50 μL): 1X reaction buffer (20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% (v/v) Triton X-100, 0.1 mg/mL BSA), 4 mM $MgSO_4$, 0.2 mM dNTPs, 0.5 pmol/μL primer I and II, respectively, 1.25 ng/μL barley α-amylase cDNA (GeneBank #J04202) and 1.25 U of Pfu DNA polymerase (Fermentas, catalog #EP0571). PCR thermal cycling conditions were set up as follows: (1) initial denaturation step at 95°C for 5 min; (2) denaturation step at 95°C for 1 min; (3) annealing step at 52°C for 30 sec; (4) extension step at 72°C for 3 min; (5) repeat 25 cycles from step (2) to step (4); (6) final extension step at 72°C for 5 min.

Barley α-amylase cDNA was obtained from Rogers, J.C. (1983). α-Agglutinin cDNA was obtained from Lipke, P. (1989).

3.3.2 Restriction Digestion of Plasmid DNA and PCR Generated DNA Fragments

Digestion of DNA (plasmid DNA or PCR generated DNA fragments) with restriction enzymes was carried out with 1 μg DNA, 2 μL of 10X buffer, and 1-2 U of each restriction enzyme in a final volume of 20 μL. Digestions were performed at 37°C for 1-2 hours.

3.3.3 Agarose Gel Electrophoresis

DNA samples (plasmid DNA or PCR generated DNA fragments) digested with restriction enzymes were subjected to agarose gel electrophoresis on a 1% agarose gel containing 40 mM Tris-Acetate and 1 mM EDTA (TAE) at pH 8.0 and 1 μg/mL ethidium bromide in TAE running buffer. DNA samples were mixed with an appropriate volume of 5X agarose gel sample buffer (New England, Biolabs), before being loaded onto the gel. Electrophoresis was carried out at 85 volts until the necessary resolution was achieved.

DNA samples were purified from the agarose gel by using the Qiagen Gel Purification Kit (Qiagen).

3.3.4 Ligation of Digested Plasmid DNA and PCR DNA Fragments

Generally, ligation reactions were performed in two different ways depending on the ends of the DNA fragments. For blunt-ended DNA fragments, each reaction was set up as follows: 15-60 fmol of vector DNA, 45-180 fmol of insert DNA, and 1 unit of T4 DNA ligase were used for each 20 μL reaction, and the reaction was performed at 16ºC overnight. For cohesive-ended DNA fragments, each reaction was set up as follows: 3-30 fmol of vector DNA, 9-90 fmol insert DNA and 1 unit of T4 DNA ligase were used for each 20 µl ligation reaction and the reactions were performed for either 1-3 hours or overnight at room temperature. For each ligation reaction, the total DNA used was less than 0.1 µg, and the insert:vector molar ratio was adjusted to 3:1. Normally, for each given ligation, a background control ligation reaction was performed by replacing the insert DNA in the reaction mixture with water.

3.3.5 Competent Bacteria Preparation

A single colony of *E. coli* NM522 was picked from a LB plate and inoculated into 2 mL of LB medium, then incubated with shaking at 180 rpm at 37ºC overnight. The 2 mL overnight

culture was transferred into another 200 mL of sterilized LB medium, followed by an additional 1-2 hour growth with shaking at 200 rpm 37ºC. The bacterial cultivation was stopped when the $O.D._{590}$ reached 0.375. The cell culture was transferred into pre-chilled sterile 50 mL tubes, and centrifuged at 7,000X g at 4ºC for 7-10 min to collect the cells. The cell pellet was resuspended in 10 mL of cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, and 10 mM PIPES pH 7.0). The washing step was repeated twice and the cell suspension was kept on ice for 30 min followed by a further centrifugation step to re-pellet the cells. The cell pellet was resuspended in 4-8 mL of cold $CaCl_2$ solution. The concentrated cell suspension was aliquoted into 1.5 mL sterile tubes and stored at -80ºC.

3.3.6 *E. coli* Transformation

Competent *E. coli* NM522 cells were thawed on ice. One hundred μL of competent cells were mixed and aliquoted into pre-chilled 14-mL BD Falcon polypropylene round-bottom tubes. DNA ligation mixtures were mixed with competent cells by gently swirling the tube several times. The tubes were incubated on ice for 30 min, then subjected to heat-pulse at 42ºC for 2 min. The tubes were kept on ice for at least 1 min after heat-pulse. The entire transformation mixture from each tube was spread onto a LBA plate under sterile conditions, and the plates were incubated at 37ºC overnight.

3.3.7 Mini-Preparation of Plasmid DNA

Selected colonies were inoculated into 5 mL of TB medium with the appropriate amount of antibiotic, and incubated with shaking at 37ºC overnight. Plasmid was isolated by the alkaline lysis method described by Sambrook *et al.* (1989). Plasmid DNA was resuspended in a solution of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0). The sequence of plasmid DNA was confirmed by DNA sequencing. The α-amylase expression plasmid construct was named as pAMY. Also, a control plasmid, pSAC, was constructed by deleting part of barley α-amylase cDNA in pAMY through a double *Sac II* enzyme digestion. Samples were stored at -20ºC or directly used for restriction enzyme digestion.

3.4 Yeast Transformation and Selection

Protocols in this section are modified versions of those described in Sambrook *et al.* (1989) and the lithium transformation method from Invitrogen (catalog #V510-20, version F 5-20-2003).

3.4.1 Preparation of Competent Yeast

An entire yeast colony was picked from a YPD plate, transferred into 25 mL of YPD medium, and cultivated with shaking at 230 rpm at 30°C overnight. The cell culture was gradually transferred into 300 mL of fresh YPD medium until the $O.D._{600}$ reached 0.4. The fresh culture was allowed to grow for another 3 hours with shaking at 230 rpm at 30°C. The cells were then pelleted at 3000X g for 5 min and washed once with 25 mL of sterile double-distilled water. After re-pelletting, cells were resuspended in 1 mL of 1X TE/LiAc solution (100 mM lithium acetate, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and used immediately for transformation.

3.4.2 Yeast Transformation

For each transformation, 1 μg of plasmid DNA, 100 μg denatured sheared salmon sperm DNA (Sigma-Aldrich, catalog #D9156) and 100 μL of the yeast suspension in 1X TE/LiAc solution were added into a 13-mL Falcon tube. Six hundred microlitres of PEG/LiAc solution (40% PEG-3350 solution, 100 mM lithium acetate, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added to the mixture and mixed by brief vortexing. The yeast culture was then placed in a shaking incubator at 230 rpm at 30°C for 30 min. After incubation, 70 μL of DMSO was added and the mixture was subjected to heat-pulse at 42°C for 15 min. Cells were pelleted by centrifugation at 14,000X g for 15 sec in a microcentrifuge. The collected cells were resuspended in 500 μL of 1X TE buffer (10 mM Tris-HCl, and 1 mM EDTA, pH 8.0). Cell suspension was plated on YPD plates containing the indicated concentration of blasticidin (see below).

3.4.3 Determination of the Appropriate Concentration of Blasticidin to Use for Selection

The same amount of transformed yeast cells or wild type yeast cells was plated on a group of YPD plates that contained increasing concentrations of blasticidin ranging from 0 to 100 μg/mL. The plates were incubated at 30°C for 2 days, and the number of colonies on each plate was recorded for comparison. The blasticidin concentration was determined based on the disappearance of wild type yeast colonies, and the ability of transformed yeast to produce colonies.

3.5 α-Amylase Assays

3.5.1 Detection of α-Amylase Activity on Starch Containing YPD Plate Using Iodine Vapour

Transformed yeasts were incubated on YPD agar plates containing 1% soluble potato starch (Sigma-Aldrich, catalog #S4251) at 30°C for 2-3 days. When the plates were prepared, potato starch powder was first added into the YPD-agar media and then autoclaved. The starch-containing YPD-agar solution was poured into plates and solidified at room temperature. Blasticidin was added to 100 μg/mL. Transformed yeast cells were plated on the starch-containing YPD plates, at a dilution (approximately 1 in 1000) that gave colonies that were well-separated from each other after 3 days incubation at 30°C. With clearly visible colonies, the plates were stained with iodine vapour. The staining procedure was performed based on that described by Steven *et al.* (1984). Iodine crystals (I_2) vaporize at room temperature. Iodine crystals were placed into a beaker in a fume hood, with the circumference of the beaker matching that of the plate. The plate was inverted onto the beaker, exposing the colonies to the vaporized iodine. Staining was considered complete when the medium on the plate turned a dark purple colour. Pictures were taken with a digital camera when the staining was complete.

3.5.2 Quantification of Cell Surface Anchored α-Amylase Activity by the 3, 5-Dinitrosalicylic Acid (DNS) Assay and the Iodine Assay

The DNS assay kit was purchased from Sigma-Aldrich (catalog #A2771). The colour reagent contains sodium potassium tartrate solution (5.3 M sodium potassium tartrate, tetrahydrade, Sigma-Aldrich, catalog #S2377 in 2 M NaOH) and 3, 5-dinitrosalicylic acid solution (96 mM 3, 5-dinitrosalicylic acid, Sigma-Aldrich, catalog #D0550 in 20 mL of deionized water). The solutions were prepared according to the manufacturer's instructions.

The iodine colour reagent contained iodine (1.5% (w/v) I_2, Sigma-Aldrich, catalog #229695) and potassium iodide (5% (w/v), KI, Sigma-Aldrich, catalog #204102). The solution was prepared as described in Wilson *et al.* (1982).

The 1% starch solution used as the substrate for the α-amylase reaction was prepared by adding 1 g potato starch powder to 100 mL of 16 mM sodium acetate buffer (pH 4.5), and boiling for 15 min with constant stirring. After the solution cooled to room temp, water was added to bring the solution up to 100 mL.

Yeast were cultivated in a 250-mL shake flask containing 100 mL of YPD media with shaking at 200 rpm at 30°C until $O.D._{600}$ reached 0.9 to 1. Cells were harvested by centrifugation at 4000X g for 5 min at 4°C. The cell pellet was washed twice with 16 mM sodium acetate buffer (pH 4.5), then resuspended in 3 mL of sodium acetate buffer (pH 4.5). When required, blasticidin was added to the medium to a final concentration of 100 μg/mL.

Three mL of concentrated yeast cell suspension was mixed with 1 mL of 1% starch solution. The reaction mixture was placed in a 45°C water bath and samples were taken every hour over a 6 hour period. The reaction mixtures were re-mixed before each sample was taken. Cells were collected by centrifugation at 12,500X g for 1 min. Three hundred μL of supernatant were carefully transferred into a test tube with the addition of 1 mL of DNS colour reagent for the DNS assay or into a test tube with the addition of 2 mL of iodine colour reagent for the Iodine assay.

For the DNS assay, the test tube was placed in a boiling water bath for 15 min. After boiling, the sample was diluted with 9 mL of water. Absorbance was determined using a spectrophotometer at 540 nm. The blank was treated in the same way as the samples except that 3 mL of water instead of 3 mL of cell suspension were mixed with 1 mL of 1% starch

solution. For the Iodine assay, the sample was diluted in 9 mL of water before absorbance was determined using a spectrophotometer at 580 nm. The blank was treated in the same way as the samples except that 3 mL of water instead of 3 mL of cell suspension was mixed with 1 mL of 1% starch solution.

3.6 Batch Fermentation

3.6.1 Seeding Cultures

The yeast strains used in batch fermentation studies were wild type NRRL Y-132 and pAMY harbouring NRRL Y-132 (NRRL Y-132/pAMY). The cell cultures used as the seeding cultures to inoculate batch fermentations were prepared as follows: 1 mL of frozen cell culture was added to 100 mL of fresh yeast synthetic media in a 250 mL-shake flask. The culture was incubated on a shaking incubator at 150 rpm at room temperature overnight. When NRRL Y-132/pAMY was cultivated, 100 μg/mL blasticidin was added to the broth.

Frozen cultures were prepared by picking a single colony with a sterilized needle, and transferred into a 50-mL sterilized Falcon tube containing 10 mL of fresh yeast synthetic media. The culture was incubated with shaking at 180 rpm and 37°C overnight. The next day, the overnight culture was transferred into a 250 mL-shake flask containing 200 mL of fresh yeast synthetic media. The cell culture was incubated with shaking at 180 rpm at 37°C until the $O.D._{600}$ reached 0.9 to 1.0, and then 930 μL of the cell culture was transferred into a 1.5-mL sterilized microcentrifuge tube along with 70 μL of DMSO. The tubes were stored at -80°C.

3.6.2 Bioreactor Conditions and Media Composition

Fermentation conditions were as follows: 100 mL of seeding culture was added to 1.9 L fresh medium to give a total reaction volume of 2 L. The fermentation process was carried out in a bioreactor (New Brunswick Scientific model 2.5-L Bioflo 310 fermentor) at 30°C with the agitation rate at 375 rpm and airflow rate of 0.02 L/min. When D-glucose was used as the carbon source, 1.9 L of yeast synthetic media containing and 2% (w/v) D-glucose was combined with the seeding culture into the medium at the beginning of batch fermentation.

When soluble starch was used as the carbon source, 1.9 L fresh medium was composed of 1.1 L of yeast medium and 800 mL of 2% (w/v) soluble wheat starch.

The source of the soluble wheat starch was Wheat Starch 4 donated by CSP Mills, Saskatoon (manufactured by Archer-Daniels-Midland/Ogilvie in Montreal). Wheat starch 4 is a class of A grade starch which consists of particles ranging from 18 to 20 μm in size. Forty gram of starch particles were weighed and transferred into a 400 mL-shake flask and mixed with 200 mL of deionized water. The slurry of starch-water mixture was slowly added into 400 mL of boiling water with constant stirring. One hundred mL of deionized water was used to wash the starch residues left in the flask. The starch solution was boiled for another 15 min with constant stirring. After the solution had cooled to room temperature, it was brought to 800 mL with deionized water.

3.6.3 Starch Hydrolysing Enzymes

Barley malt α-amylase (α-1,4 glucan glucanohydrolase, EC 3.2.1.1, type VIII-A) (catalog #A-2771) and glucoamylase (amyloglucosidase; 1, 4, α-D-glucan glucanohydrolase, EC 3.2.1.3) (catalog #A-7255) from *Rhizopus* mold were obtained from Sigma-Aldrich. In some batch fermentation experiments, the amount of 15 g/L barley α-amylase and/or 1 g/L glucoamylase was added into the fermentor 30 min prior to inoculation.

3.6.4 Biomass Analysis

Biomass at certain time point was recorded as either $O.D._{600}$ or total cell number, depending on the viscosity of fermentation broth caused by soluble starch.

When glucose was used as sole carbon source or soluble starch as sole carbon source with the addition of amylolytic enzymes, biomass was initially recorded as $O.D._{600}$. Samples were taken from the fermentor and measured on spectrophotometer at 600 nm. Dilutions were made when $O.D._{600}$ exceeded 1.0. Recorded $O.D._{600}$ was converted to biomass concentration (dry weight basis) using a previously constructed standard curve.

When soluble starch was used as sole carbon source, direct measurement of biomass through spectrophotometer was impossible due to high viscosity of the fermentation broth caused by soluble starch. Therefore, biomass was recorded as total cell number by counting the cell number of a sample through a Petroff Hauser hemocytometer under a microscope. The cell

number counted in the sample was converted into the total cell number in the fermentor. The sample applied on the Petroff Hauser hemocytometer was generally diluted 11 fold from the original sample taken from the fermentor to give clearly separated cells. Normally, cell number was counted in 5 to 6 separate squares on the Petroff Hauser hemocytometer, and an average of these numbers was used to calculate the total cell number.

The equation used was: Total cell number in 2-L fermentation broth = Average cell number × 11 (dilution factor) × 16 (number of the smallest squares in each square used for counting) × 1/400 mm^2 × 1/50 mm (the last two values give the volume of each smallest square indicated on the Petroff Hauser hemocytometer).

3.6.5 Ethanol Analysis

Two different methods were used to collect samples for ethanol analysis depending on the viscosity of fermentation broth caused by soluble starch.

When glucose was used as sole carbon source or soluble starch as sole carbon source with the addition of amylolytic enzymes, samples were taken from the fermentor and filtered through a 0.2 micron filter to produce a clear filtrate for ethanol analyses. Filtrates were stored in 1.5-mL Agilent vials (Agilent Technology, U.S.) at -20°C.

When soluble starch was used as sole carbon source, direct filtration of fermentation broth through 0.2 micron filter was impossible due to high viscosity of the fermentation broth caused by soluble starch. Therefore, the sample collected from the fermentor was pre-treated with a small amount of barley α-amylase in a sealed container to hydrolyse the starch prior to filtration. A detailed description of the procedure is given below.

During fermentation, 15 mL samples were collected from the fermentor and placed into sterilized 50-mL Falcon tubes. Cells were pelleted by centrifuging at 3000X g for 3 min and the supernatant was transferred into a glass container and mixed with approximately 10 μg of barley α-amylase. The container was sealed and held at room temperature for 5 min. The supernatant was then filtered through a 0.2 micron filter. Filtrates were stored in 1.5-mL Agilent vials at -20°C.

Ethanol concentrations were determined by gas chromatography using a flame ionization detector and a 30 m, 0.25 mm ID poly (5% phenyl, 95% dimethyl) siloxane capillary column. The oven temperature was 60°C and 1-butanol was used as an internal standard (Lang

et al., 2001). Frozen samples were thawed at room temperature. Samples were placed on the sample region of the detector in order and injected automatically into the detector. For each sample, three separate injections were analysed.

The theoretical maximum yield of ethanol from 100 parts of glucose is 51.1 (Ingledew, 1993). The fermentation efficiency can be calculated as:

% fermentation efficiency = (weight of ethanol produced × 100)/(theoretical weight of ethanol from produced glucose)

3.6.6 Soluble Starch Concentration

The method used to measure the soluble starch concentration in frozen samples collected from batch fermentation was modified from the Iodine assay method (section 3.5.2).

Frozen samples were first boiled for 30 min. in a water bath. Then 100 μL of samples were mixed with 100 μL of deionized water and 2 mL of Iodine Colour Reagent (5 mM I_2 and 5 mM KI). Nine mL of water was added before measuring absorbance at wavelength 580 nm using a spectrophotometer. The soluble starch concentration in the sample was calculated from a previously constructed standard curve.

3.6.7 Plasmid Stability

Cell samples were collected from the fermentor during batch fermentation, and diluted to the appropriate cell density with sterilized deionized water. Equal amounts of cell suspension were plated on both YPD plates and blasticidin-containing YPD plates. For each sample set, three different dilutions were prepared to ensure that one dilution would give the appropriate cell density such that the colonies on the plates were separate from each other for accurate counting. For each dilution, three YPD plates and three blasticidin-containing YPD plates were plated to improve statistical accuracy. Plates were incubated at 30°C for 1 to 2 days. After counting the number of colonies, the ratio of the number of colonies on blasticidin-containing YPD plates over that on YPD plates was recorded as relative plasmid stability.

4.0 RESULTS

4.1 Construction of Novel Recombinant Plasmid pAMY

Naturally occurring yeast strains are unable to utilize starch as carbon sources for growth and fermentation because they lack the ability to hydrolyse starch. Therefore, a novel recombinant yeast strain was designed and constructed from an industrial ethanol-producing yeast strain, *S. cerevisiae* NRRL Y-132. This novel recombinant strain was designed to express and anchor a starch-hydrolysing enzyme, barley α-amylase on its cell surface, so that the novel strain is able to perform simultaneous starch hydrolysis and fermentation. As detailed in section 2.4, functional α-amylases from various sources have been reported to be expressed in yeast. Barley α-amylase was used in this study, since in cold-starch hydrolysis fermentation, barley α-amylase was shown to be superior to α-amylases from other sources due to its optimal kinetics at the temperature and pH that is compatible with that needed for yeast growth and fermentation (Lim *et al.*, 2003).

The first goal of my study was to construct a fusion gene composed of a barley α-amylase cDNA that had an oligonucleotide coding for a secretion signal sequence attached to its 5' end and its 3' end fused to the 3' half of the α-agglutinin cDNA. The complete fusion gene was inserted into the MCS of yeast episomal plasmid pSCW231 at restriction sites *EcoR I* and *Kpn I* with constitutive promoter *ADH1* at an upstream site and a transcription termination sequence, *CYC1*, at a downstream site (Figure 4.1).

The original yeast selection marker carried by pSCW231, *Trp*$^-$, belongs to a group of auxotrophic markers which are used to select genetically-engineered yeast strains that are transformed with a plasmid carrying the auxotrophic marker gene. However, the studied yeast strain, *S. cerevisiae* NRRL Y-132, is an ethanol producing strain that is used in the ethanol industry and, in this case, cannot be selected by using auxotrophic markers because there is no corresponding auxotrophic mutant. Blasticidin is an antibiotic that inhibits the growth of a wide range of prokaryotic and eukaryotic cells by interfering with their protein synthesis (Invitrogen, Catalog #R210-01). Blasticidin specifically inhibits protein synthesis through

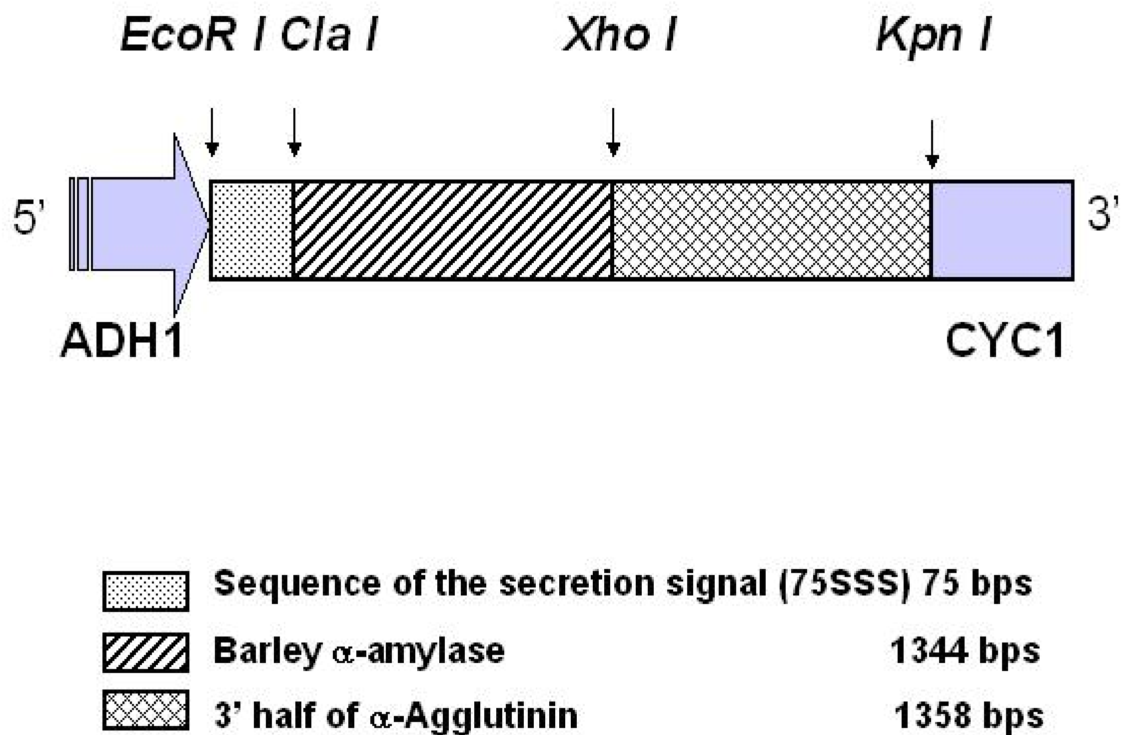


Fig. 4.1 Structure of the Fusion Gene. The fusion gene is composed of a contiguous sequence of the secretion signal of glucoamylase precursor protein (75SSS), complete cDNA of barley α-amylase and the 3' half of the α-agglutinin cDNA. Transcription of the fusion gene is under control of the ADH1 promoter. The restriction sites used to construct the fusion gene are indicated at their corresponding positions.

inhibition of peptide-bond formation in the ribosomal machinery (Izumi *et al.*, 1991). Yeast cells that are not transformed with a blasticidin resistance gene will not survive in the media containing an appropriate concentration of blasticidin. Thus, a blasticidin resistance gene was inserted into the plasmid. The complete structure of pAMY is shown in Figure 4.2. Also, a control plasmid, pSAC, was constructed by deleting part of barley α-amylase cDNA in pAMY through a double *Sac II* enzyme digestion. The DNA sequence of pAMY was confirmed by DNA sequencing.

4.2 Recombinant Yeast with Cell Surface Anchored Barley α-Amylase

S. cerevisiae NRRL Y-132 was transformed with pAMY by performing a lithium acetate yeast transformation (section 3.5.2). A general description of the fusion gene expression, cell surface anchoring of the fusion protein, and the pAMY harbouring yeast (NRRL Y-132/pAMY) as a whole cell catalyst for starch hydrolysis and fermentation is the objective of this section. The fusion gene (75SSS/α-amylase/3' half of α-agglutinin) was constitutively expressed in NRRL Y-132/pAMY. As detailed in section 2.3, the fusion protein is recognized as a cell wall-targeted protein inside the ER. Following cleavage of the signal peptide 75SSS, the remaining part of the fusion protein (barley α-amylase and C-terminal half of α-agglutinin) would be transferred to a GPI anchor and transported to the cell surface. When it reaches the cell surface, it is believed that the GPI attached protein is transferred from the GPI anchor to the glucan of the cell wall, with the C-terminal end of α-agglutinin covalently linked to the glucan structure and barley α-amylase facing into the medium. A diagram of NRRL Y-132/pAMY with cell surface-anchored barley α-amylase is shown in Figure 4.3.

4.3 Determination of the Appropriate Concentration of Blasticidin to Use for Selection

In order to identify positive yeast clones harbouring pAMY, the appropriate concentration of blasticidin used for selection had to be determined. For comparison, two other yeast strains were used as controls: NRRL Y-132 transformed with control plasmid pSAC and wild type NRRL Y-132. The same amount of cell suspension of each strain was plated on YPD

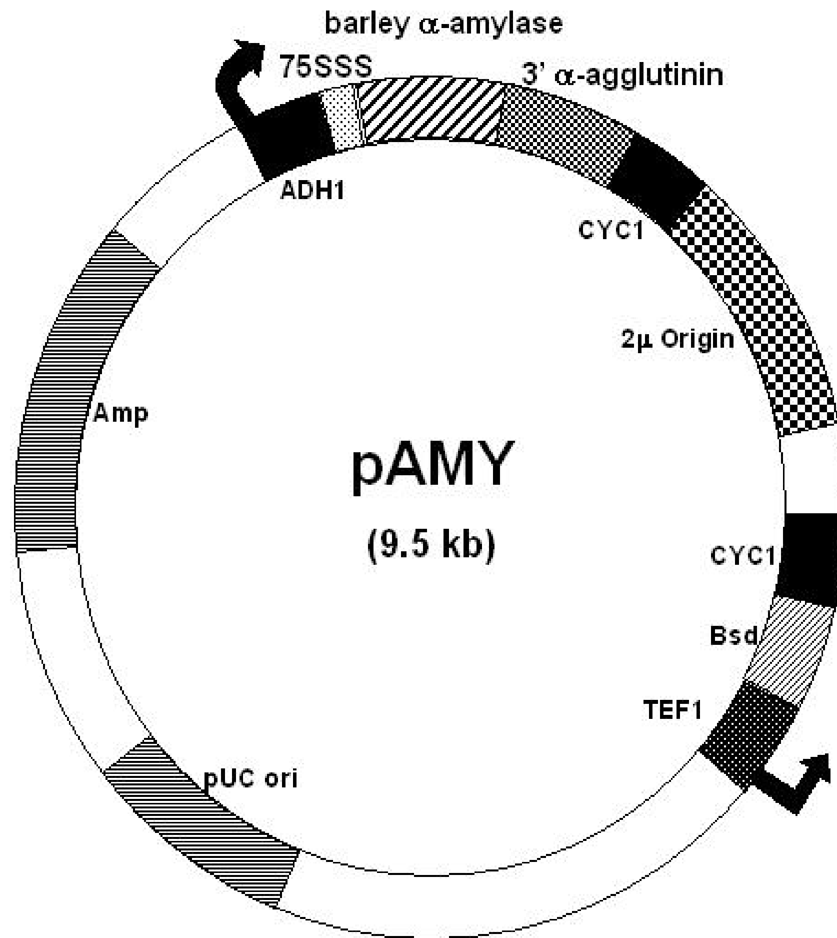


Fig. 4.2 The Structure of Yeast Expression Plasmid pAMY. The plasmid was designed for cell surface expression of the barley α-amylase/3' half of the α-agglutinin fusion gene. It contains a constitutive promoter *ADH1*, a transcription termination sequence, *CYC1*, an ampicillin resistance gene for selection in *E. coli*, a 2μ origin sequence that keeps high copy numbers of the plasmid inside yeast cells and a blasticidin resistance (Bsd) gene as a selectable marker for selection in yeast, which is under the control of a constitutive yeast promoter *TEF1*. Arrows are placed at each promoter region to indicate transcription directions.

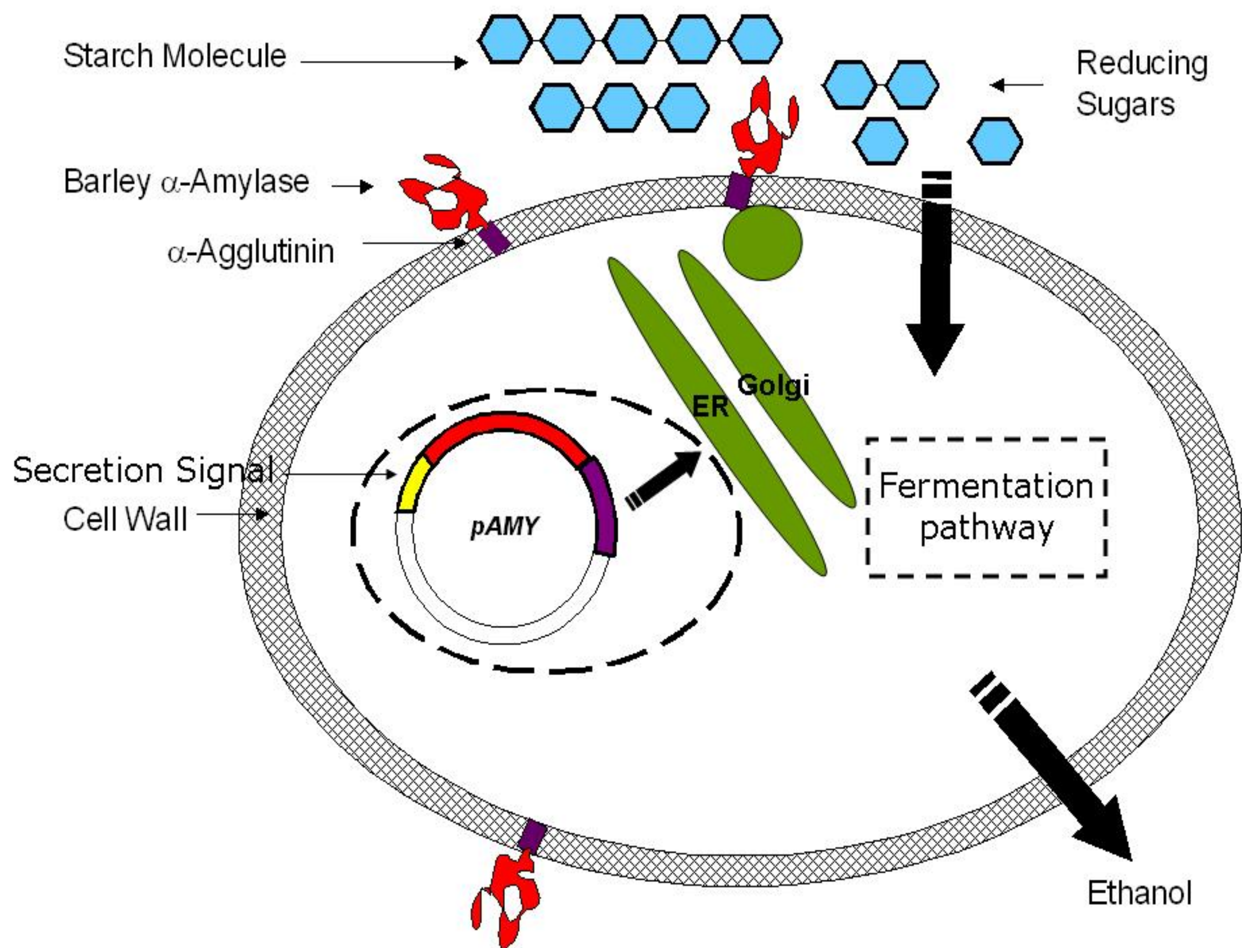


Fig. 4.3 Model of α-Amylase Expressing Recombinant Yeast. NRRL Y-132/pAMY expresses cell surface anchored barley α-amylase and is able to perform starch hydrolysis and fermentation. After expression, the secretion signal (indicated as yellow bar in pAMY) is cleaved in ER and the rest of the fusion protein is transported to the cell surface through Golgi. The sizes of the cell wall anchored proteins and starch molecules are scaled up in the picture to give a better demonstration of the mechanism.

plates containing blasticidin concentration ranging from 0 to 100 μg/mL, respectively. The number of colonies of each strain that grew on the YPD plates containing the same amount of blasticidin was counted and compared (Figure 4.4). In the absence of blasticidin, the number of colonies from each of the 3 strains was enough to cover the entire YPD plates. Wild type NRRL Y-132 colony formation was significantly reduced at blasticidin concentration of 20 μg/mL and no colonies were detected at 60 μg/mL. Colony formation of yeast harbouring pAMY and pSAC were also reduced at blasticidin concentration of 20 μg/mL, but to a much less extent comparing to wild type NRRL Y-132. In addition, this reduction was not significantly affected by increasing blasticidin concentrations up to 100 μg/mL. Based on these observations, a blasticidin concentration of 100 μg/mL was determined to be the appropriate concentration to select NRRL Y-132/pAMY and NRRL Y-132/pSAC, and was used for culturing all of the transformed yeast cells in subsequent studies.

4.4 Detection of α-Amylase Activity in NRRL Y-132/pAMY Using Iodine Vapour Approach

Blasticidin resistant yeast were subjected to a starch plate assay to detect whether they expressed functional barley α-amylase. Iodine complexes with starch, resulting in dark purple staining. If the pAMY harbouring yeast expressed functional cell surface anchored barley α-amylase, white haloes can be visualized around each colony formed on a starch plate after staining with iodine vapour due to amylolytic degradation of the surrounding starch (Filho *et al.*, 1986; Ruohonen *et al.*, 1987; Kondo *et al.*, 2002).

As detailed in section 3.7, the assay was performed using iodine vapour applied to yeast growing on YPD agar plates containing soluble starch. As shown in Figure 4.5A and 4.5B (left panel), haloes were detected around each yeast colony harbouring pAMY after iodine vapour staining. In contrast, yeast colonies harbouring control plasmid pSAC, which has the barley α-amylase cDNA removed, showed no halo formation (Figure 4.5B, right panel). This indicated that the yeast harbouring pAMY expressed functional barley α-amylase.

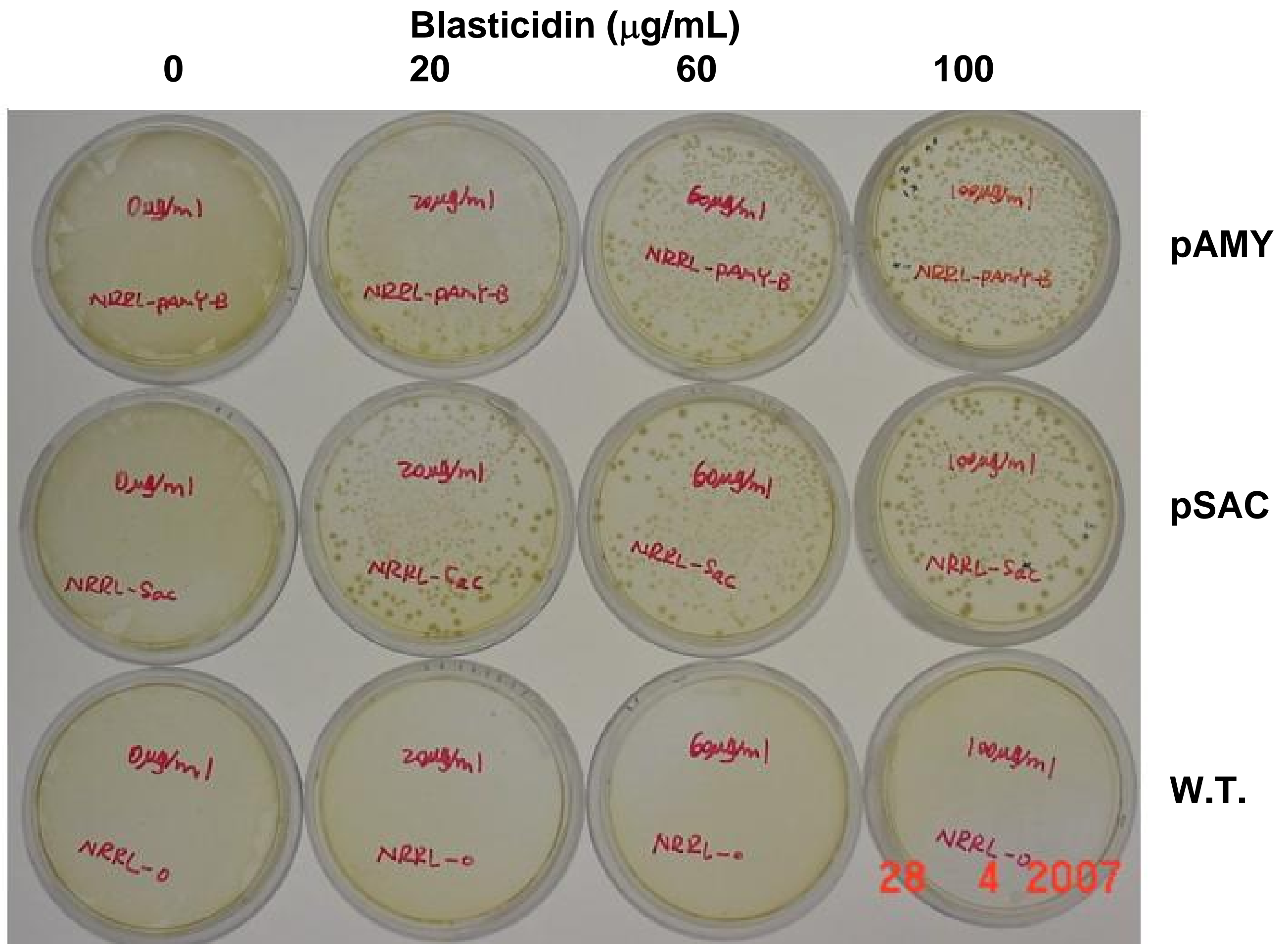


Fig. 4.4 Cell Growth Response to Various Blasticidin Concentrations on YPD Agar. The pAMY harbouring (indicated as pAMY), pSAC harbouring (indicated as pSAC) and wild type (indicated as W.T.) *S. cerevisiae* NRRL Y-132 cell suspensions with the same cell density were plated on YPD agar plates with various blasticidin concentrations as indicated above. Colonies became clearly visible after 2 days of incubation at 30°C. A representative result from four independent experiments is shown.

Fig. 4.5 Detection of α-Amylase Activity in NRRL Y-132/pAMY Using Iodine Vapour Assay. **A.** Yeast colonies harbouring pAMY were cultured on a YPD agar plate containing 1% soluble starch. After iodine vapour staining, haloes were observed around the colonies. **B** (left panel) Enlarged view of a single colony harbouring pAMY stained by iodine vapour; (right panel) enlarged view of a single colony harbouring pSAC stained by iodine vapour for comparison.

4.5 Quantification of α-Amylase Activity with DNS and Iodine Assays

Halo formation around the colony of NRRL Y-132/pAMY cannot give quantitative information about how much α-amylase activity was actually expressed. It was my objective to investigate the possible assays that could be used to quantify cell surface anchored α-amylase activity.

Two different assays, the Iodine assay and DNS assay, were explored for their suitability. As detailed in section 2.4, both assays are widely used to quantify α-amylase activity (Miller, 1959; Xiao *et al*., 2006). Both assays have been used to measure the activity of α-amylase activity that had been expressed and secreted into the cultural medium (Filho *et al*., 1986; Ruohonen *et al*., 1987; Moraes *et al*., 1995; Murai *et al.*, 1999). Information is limited on examining their use to measure cell surface anchored α-amylase activity. Both assays are based on the principle that α-amylase breaks down starch polymers into reducing sugars, primarily maltose and glucose. The Iodine assay measures the amount of soluble starch remaining after the reaction, and thus measures the change in substrate concentration, while the DNS assay measures the amount of reducing sugars released in the reaction, and thus measures the formation of product.

The feasibility of the two assays for measuring the cell surface anchored α-amylase activity using NRRL Y-132/pAMY as the catalyst was investigated. My first objective was to examine whether the two assays generated comparable results when they were performed under the same reaction conditions. Colour development for each assay at 4 different time points is shown in Figure 4.6. With increasing incubation time, the samples subjected to the Iodine assay showed a gradual decrease in colour intensity (Figure 4.6A), while samples subjected to the DNS assay showed a gradual increase in colour intensity (Figure 4.6B). The results indicate that both the Iodine assay and DNS assay successfully measured product formation and substrate utilization, respectively, during the starch hydrolysis reaction when using NRRL Y-132/pAMY as the catalysts.

Quantitative data was obtained by measuring the absorbance of the assay solutions at wavelength 580 nm (for the Iodine assay) or 450 nm (for the DNS assay). The measured absorbance was converted into corresponding reducing sugar or starch concentrations using

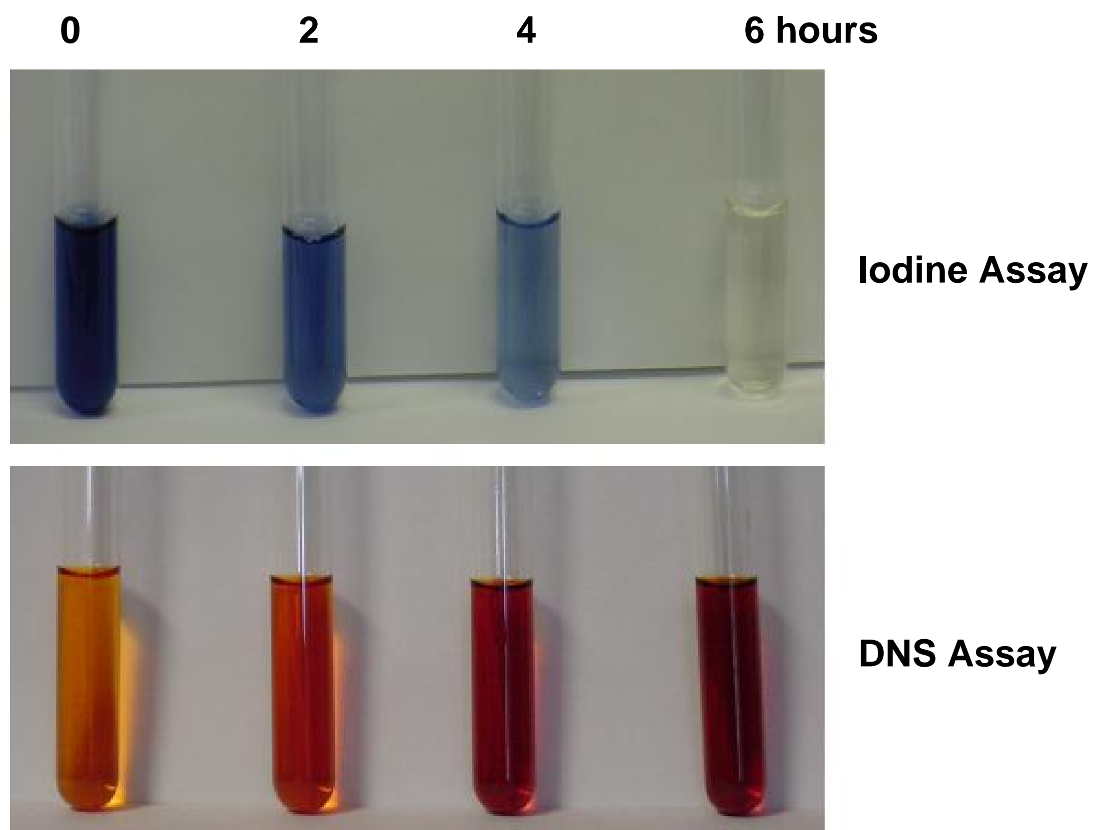


Fig. 4.6 Iodine Assay and DNS Assay for Measuring Cell Surface Anchored α-Amylase Activity. The reaction was initiated by adding a cell suspension of NRRL Y-132/pAMY into soluble starch solution at pH 4.5, 45°C. The samples were taken at the indicated time points and analysed by the Iodine assay (top) and DNS assay (bottom), respectively. This is a representative result from three independent experiments.

previously constructed standard curves. Two pAMY harbouring clones and one pSAC harbouring clone were cultivated for the assays.

In the reactions catalyzed by NRRL Y-132/pAMY, reducing sugar concentration increased linearly during the 6-hour reaction in the DNS assay (Figure 4.7), while a decrease in starch concentration was detected using the Iodine assay (Figure 4.8). In contrast, no obvious change was observed on reducing sugar and starch concentration in the control reaction containing NRRL Y-132/pSAC. The results indicate that the soluble starch was hydrolysed into reducing sugars by NRRL Y-132/pAMY under the assay conditions. All the reactions started with a soluble starch concentration of 5 mg/mL. Interestingly, no starch was detected by the Iodine assay after about 4 hours, but only 4.0 (clone 1) and 3.7 (clone 2) mg/mL of reducing sugars were generated by the end of the reactions. These data suggest that there is a higher starch-hydrolysing rate than reducing sugar generating rate in the NRRL Y-132/pAMY catalyzed reactions. The specific activity of cell surface anchored α-amylase was converted into units (Table 4.1 and 4.2). The calculated units based on the Iodine assay is about 2 times higher than the one based on the DNS assay.

Furthermore, the cell surface anchoring property of the expressed α-amylase was investigated using these two assays. By assaying the cell pellet and supernatant fraction of the cell culture, respectively, it was possible to identify whether the α-amylase was indeed anchored on the cell surface (Table 4.1 and 4.2). After the cells were pelleted, no α-amylase activity was detected in the supernatant fraction of the cell cultures. This indicated that the α-amylase was anchored on the cell surface and not secreted into the medium.

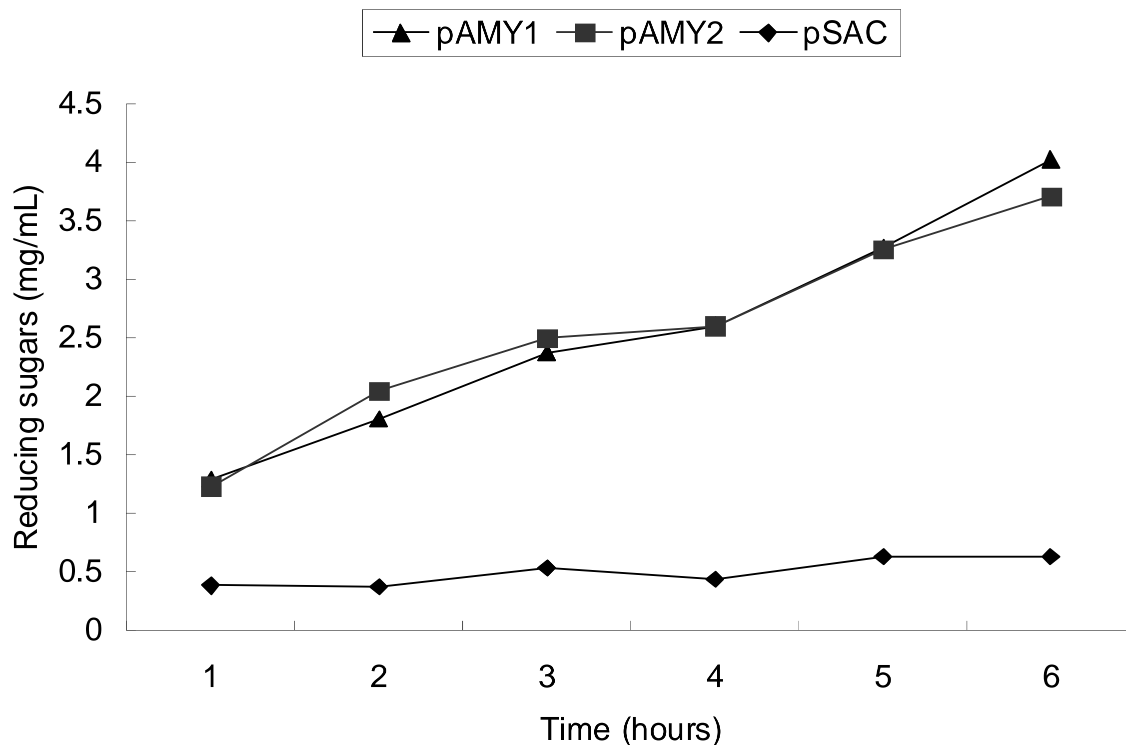


Fig. 4.7 α-Amylase Activity of Intact NRRL Y-132/pAMY Measured by the DNS Assay. The assay measured the amount of reducing sugars generated from the starch hydrolysis reaction catalyzed by NRRL Y-132/pAMY or NRRL Y-132/pSAC. The reaction was performed by mixing 3 mL cell suspension and 3 mL of 1% soluble starch and incubating at 45°C for 6 hours. Samples were taken at each hour point, centrifuged to pellet the cells, and the supernatant was used for the DNS assay. Values shown are the averages of 3 independent experiments. The standard deviations were lower than 0.004 and therefore not shown. pAMY1 and pAMY2 are both harbouring plasmid pAMY, cultured from two separate colonies from the original plate.

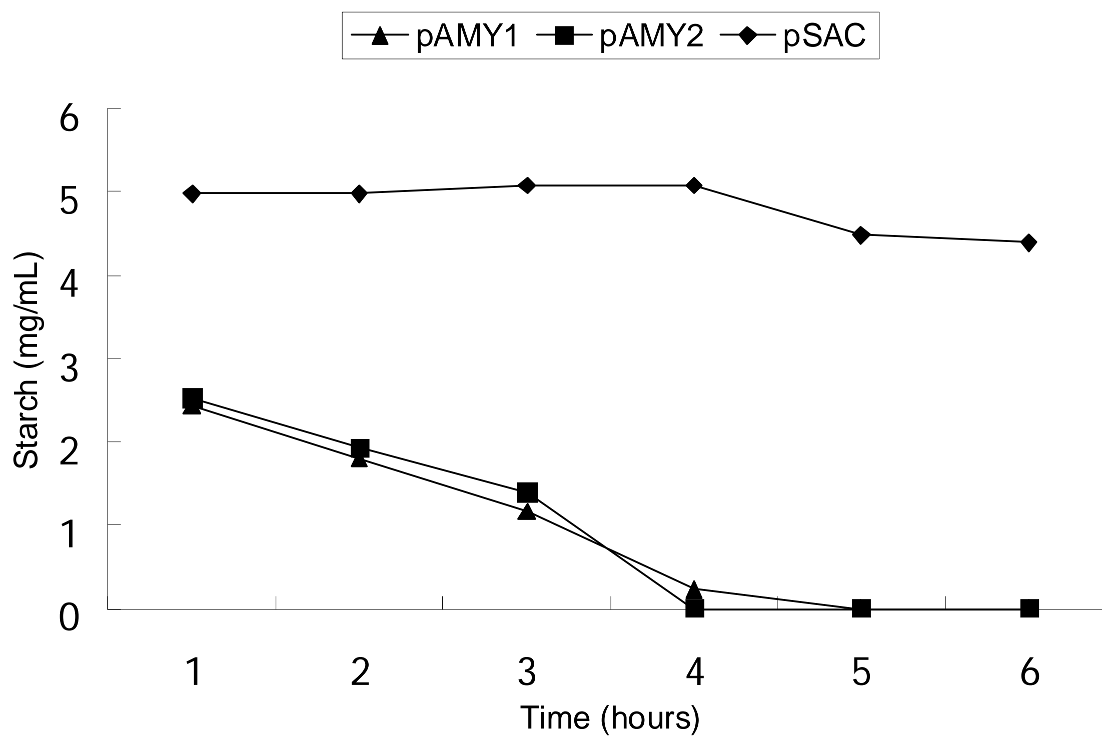


Fig. 4.8 α-Amylase Activity in Intact NRRL Y-132/pAMY Measured by the Iodine Assay. The assay measured the amount of remaining starch in the reaction catalyzed by NRRL Y-132/pAMY or NRRL Y-132/pSAC. The reaction was performed by mixing 3 mL cell suspension and 3 mL of 1% soluble starch and incubating at 45°C for 6 hours. Samples were taken at each hour point, centrifuged to pellet the cells, and the supernatant was used for the Iodine assay. Values shown are the averages of 3 independent experiments. The standard deviations were lower than 0.004 and therefore not shown. pAMY1 and pAMY2 are both harbouring plasmid pAMY, cultured from two separate colonies from the original plate.

TABLE 4.1. Quantification and Distribution of α-Amylase Activity Using the DNS Assay

Strain	α-amylase activity (U/g [wet weight] of cells)	
	Culture medium	Cell pellet
NRRL Y-132/pAMY	ND	1.3
NRRL Y-132/pSAC	ND	ND

One unit (U) of activity is defined as the amount of enzyme required to release 1 mg of reducing sugars per hour at pH 4.5, 45°C when 5 mg/mL of soluble starch was present at the start of the reaction.
ND, not detectable.

TABLE 4.2. Quantification and Distribution of α-Amylase Activity Using the Iodine Assay

Strain	α-amylase activity (U/g [wet weight] of cells)	
	Culture medium	Cell pellet
NRRL Y-132/pAMY	ND	2.5
NRRL Y-132/pSAC	ND	ND

One unit (U) of activity is defined as the amount of enzyme required to hydrolyze 1 mg of soluble starch per hour at pH 4.5, 45°C when 5 mg/mL of soluble starch was present at the start of the reaction.
ND, not detectable.

4.6 Batch Fermentation Studies

4.6.1 Batch Fermentation on Soluble Starch

The experiments above indicated that NRRL Y-132/pAMY was able to hydrolyse starch. The question about whether this strain is able to efficiently utilize soluble starch as the sole carbon source to support its own growth and fermentation activity was addressed in batch fermentation studies. First, it was necessary to determine whether the constitutive expression of α-amylase had effects on NRRL Y-132/pAMY cells' ability to proliferate and ferment. As detailed in section 3.6.2, NRRL Y-132/pAMY were inoculated into broth containing 20 g/L glucose as the sole carbon source. Biomass and ethanol production were measured as shown in Figure 4.9. Both biomass and ethanol production showed typical log shape curves, with both biomass and ethanol produced reaching their maximum values and plateauing at 15 hours. At the end of the batch fermentation, 1.9 g/L of biomass and 10.3 g/L of ethanol were produced from 20 g/L of glucose. The ethanol yield was 100% of the theoretical yield, which is comparable to the ethanol production of the wild type. This suggests that the production of anchored α-amylase did not interfere with the ability of the pAMY harbouring yeast to ferment glucose.

Next, the same amount of inoculate of NRRL Y-132 and NRRL Y-132/pAMY were used for batch fermentations under the same conditions except that 20 g/L of soluble starch was used as the sole carbon source. Samples were collected for ethanol analyses and biomass measurements over a 72-hour period (Figure 4.10A). NRRL Y-132/pAMY showed only a small amount of biomass increase on soluble starch, and no ethanol was detected for either strain (data not shown). The biomass of both wild type and pAMY harbouring yeast slowly reached a peak value at around 10 hours. Following this, the biomass of both strains continuously decreased until the end of the batch fermentations.

NRRL Y-132 was not expected to proliferate or produce ethanol when starch was used as the sole carbon source. However, since NRRL Y-132/pAMY had been shown to have starch hydrolysis ability, and considering the results that they did not perform better than NRRL Y-132 under batch fermentation with starch, it was speculated that cell death might be occurring

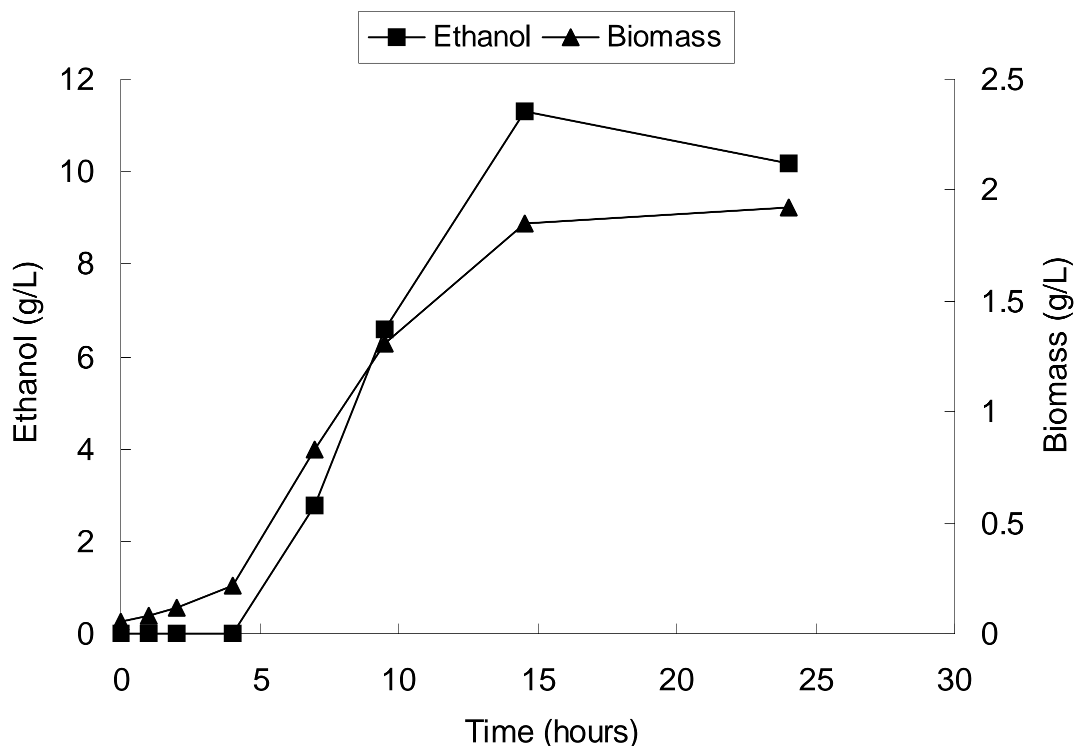


Fig. 4.9 Batch Fermentation of NRRL Y-132/pAMY on Glucose. Fermentation was carried out in a 2.5-litre bioreactor at 30°C with an agitation rate of 375 rpm and airflow at 0.02 L/min. One hundred mL of pre-cultured NRRL Y-132/pAMY was used to inoculate the fermentor. Biomass changes and ethanol production were measured over a 24-hour period at the time points indicated. NRRL Y-132/pAMY was pre-cultured with 100 µg/mL of blasticidin. This batch fermentation was performed once.

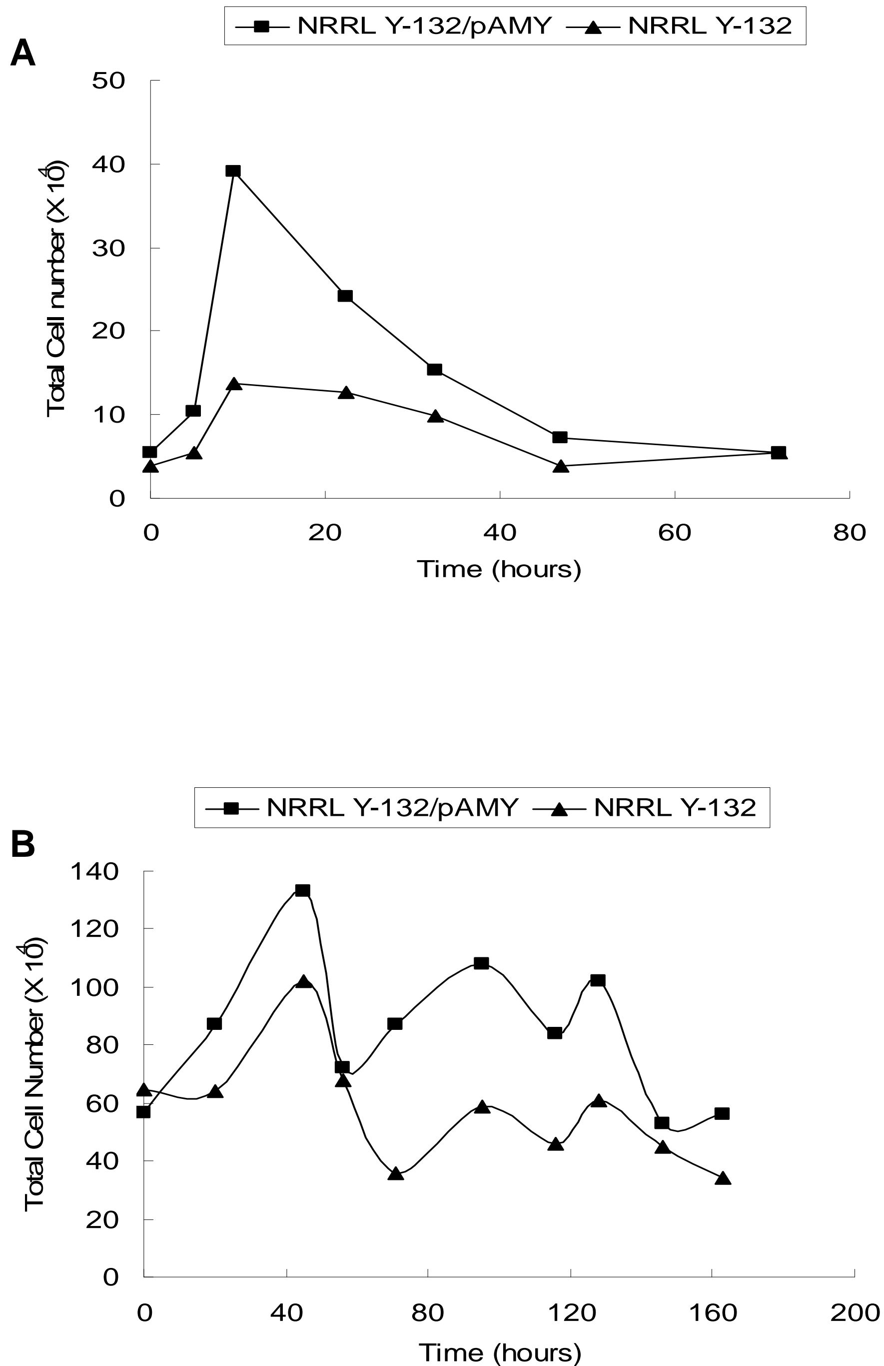


Figure 4.10 Batch fermentation of NRRL Y-132 and NRRL Y-132/pAMY on Soluble Starch. (A) One hundred mL of pre-cultured wild type or pAMY harbouring yeast was used to inoculate the fermentor. (B) Two hundred mL of cell suspension concentrated from 2 L late log phase cell culture using sodium acetate buffer at pH 4.5 was used to inoculate the fermentor. At the indicated time points, samples were collected and the number of cells was counted using a Petroff Hauser haemocytometer under a microscope. NRRL U-132/pAMY was pre-cultured with 100 µg/mL of blasticidin. Each of the batch fermentations was performed once.

due to a relative lack of α-amylase activity at the beginning of the batch fermentations. As shown earlier in Figure 4.7 and 4.8, even though NRRL Y-132/pAMY were able to perform starch hydrolysis, the availability of utilizable reducing sugars at the start of fermentation may be limiting. Therefore, a larger amount of inoculate was used to increase the initial amount of α-amylase available in the fermentor. Hence, two litres of pre-cultured pAMY-harbouring cells were concentrated into a 200-mL cell suspension using sodium acetate buffer at pH 4.5 and used as the inoculate for batch fermentations under the same conditions as in Figure 4.10A. As shown in Figure 4.10B, both NRRL Y-132 and NRRL Y-132/pAMY started at a higher initial biomass, and the biomass of both strains slowly increased to their maximum values at about 40 hours then declined. Following the peak, the biomass of NRRL Y-132 quickly declined to levels below its initial value; small increases in biomass were observed later in the fermentation, but never exceeded its initial value. In contrast, the biomass of NRRL Y-132/pAMY showed an obvious increase following the first biomass decline; an oscillation in biomass was observed later in the fermentation (Figure 4.10B). No ethanol was produced by either strain (data not shown). NRRL Y-132/pAMY was able to hydrolyse over 75% of the starch in the fermentor after 160 hours of operation, however, the wild type showed only weak starch hydrolysis ability (Figure 4.11). The results suggest that NRRL Y-132/pAMY was able to hydrolyse starch under fermentation conditions, however, at a very slow rate. Thus, the reducing sugars generated may be insufficient for cell proliferation and ethanol formation.

4.6.2 Batch Fermentation with Addition of Amylolytic Enzymes

As shown in the starch batch fermentation experiments above, even though NRRL Y-132/pAMY was able to hydrolyse soluble starch under fermentation conditions, it failed to proliferate significantly or produce ethanol. Several factors might contribute to the failure, such as reduced α-amylase activity due to cell surface anchoring, inefficient utilization of maltose by this particular yeast strain, or inhibitory effects on cell metabolism when starch is used as the sole carbon source, could cause yeast to fail to proliferate and produce ethanol. To investigate whether these factors could potentially affect NRRL Y-132/pAMY's performance during starch fermentation, several parallel starch batch fermentations using both NRRL Y-132 and NRRL Y-132/pAMY with the addition of different amylolytic enzymes were carried out.

First, the ability of wild type yeast to utilize maltose was investigated by inoculating

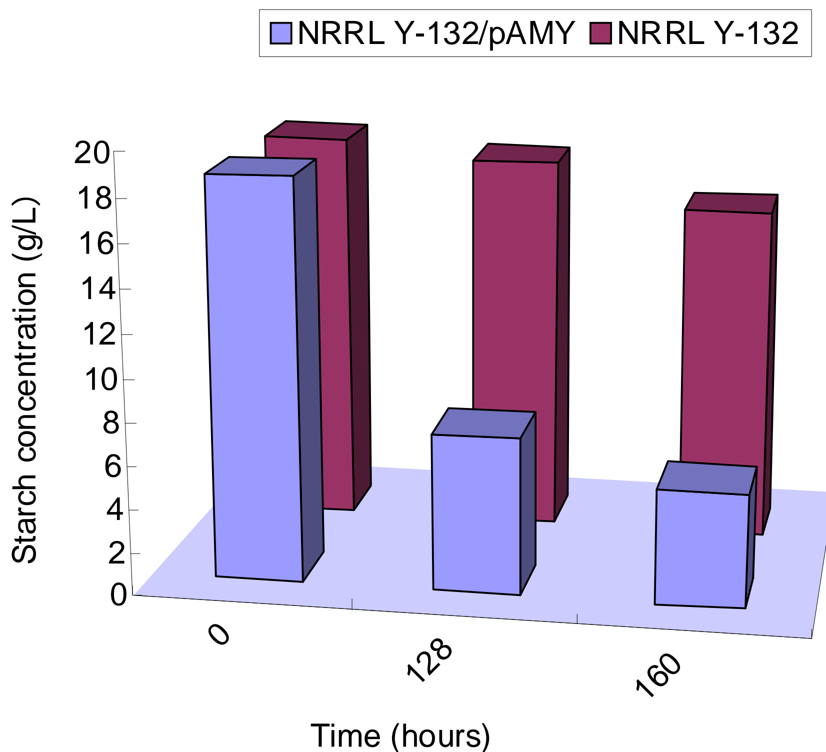


Fig. 4.11 Starch Hydrolysis in Batch Fermentation with Starch as the Sole Carbon Source. This chart shows the soluble starch concentration during the two batch fermentations showed in Figure 4.10B. Samples were taken from the fermentor at the indicated time and stored in 1.5 mL microcentrifuge tubes at -20°C. Frozen samples were boiled in a water bath for 30 min and then analysed by the Iodine assay. Absorbance at 580 nm was measured and the readings were converted to starch concentrations using the standard curve shown in Figure 4.15. The values are the average of 2 independent experiments. The standard deviations were lower than 0.004 and therefore not shown.

100 mL pre-cultured NRRL Y-132 into the fermentor medium containing 2% soluble starch and 15 g/L of barley α-amylase. Starch concentration, biomass and ethanol production were measured (Figure 4.12). Most of the starch was hydrolysed in the first hour (data not shown). Over 24 hours of fermentation, 10.6 g/L of ethanol and 2.0 g/L of biomass were generated with ethanol production being close to 100% of the theoretical yield. The results are comparable with the batch fermentation data obtained using 2% glucose (Figure 4.9). The results indicate that NRRL Y-132 is able to utilize maltose for cell proliferation and fermentation as efficiently as glucose.

The availability of utilizable reducing sugars can affect the proliferation and ethanol production of NRRL Y-132. In order to investigate this possibility, glucoamylase was used to perform starch hydrolysis in batch fermentation. As stated in section 2.3, glucoamylase hydrolyses starch polymers at a relatively slow rate compared to α-amylase and generates glucose residues. The fermentation was carried out under the same conditions as above except that 1 g/L of glucoamylase was added instead of α-amylase. The small amount of glucoamylase was used since this is the typical amount used in industrial fermentations, and to allow starch hydrolysis to occur at a relatively slow rate compared to the one performed with α-amylase. Ethanol was detected after 8 hours, and over a 36-hour fermentation, 6.1 g/L of ethanol and 1.3 g/L of biomass were produced (Figure 4.13A). The yield of ethanol and biomass was relatively low compared to the fermentation with added α-amylase. All of the starch was hydrolysed by the end of the fermentation (data not shown).

For comparison, a parallel batch fermentation was performed with NRRL Y-132/pAMY (Figure 4.13B). Over the 36 hours of fermentation, 6.8 g/L of ethanol and 2.0 g/L of biomass were produced, which are comparable to the results obtained from the batch fermentation using NRRL Y-132 under the same conditions (Figure 4.13A). Interestingly, for the batch fermentation using NRRL Y-132/pAMY, no detectable ethanol was produced in the first 23 hours, which was 15 hours delayed compared to NRRL Y-132 (Figure 4.13B). The results suggest that the yield of biomass and ethanol were compromised when the availability of utilizable reducing sugars was limiting.

In order to further confirm this, batch fermentation with the addition of both α-amylase (15 g/L) and glucoamylase (1 g/L) was performed, which would be expected to release glucose residues from starch polymer at a very fast rate. Over 24 hours of fermentation, 10.2 g/L of

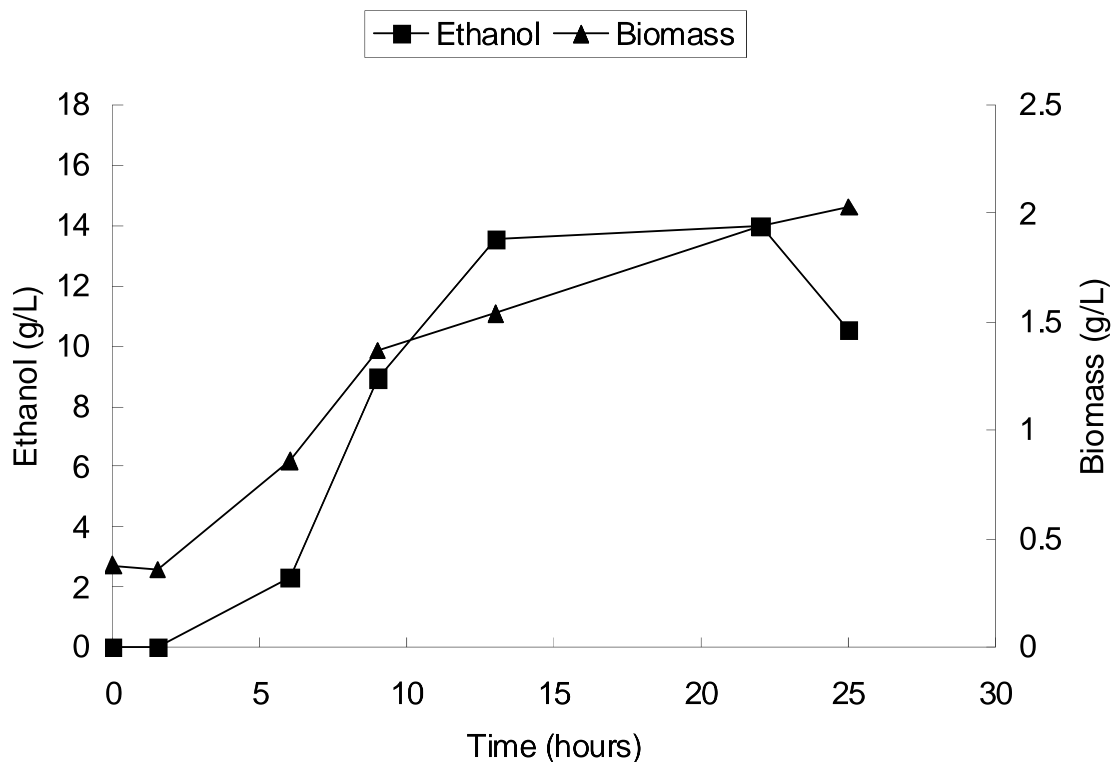


Fig. 4.12 Batch Fermentation of NRRL Y-132 with Addition of 15g/L of Barley α-Amylase. One hundred mL of pre-cultured NRRL Y-132 was used to inoculate the fermentor containing 2% soluble starch. Biomass changes and ethanol production were measured over 24 hour period at the time points indicated. This batch fermentation was performed once.

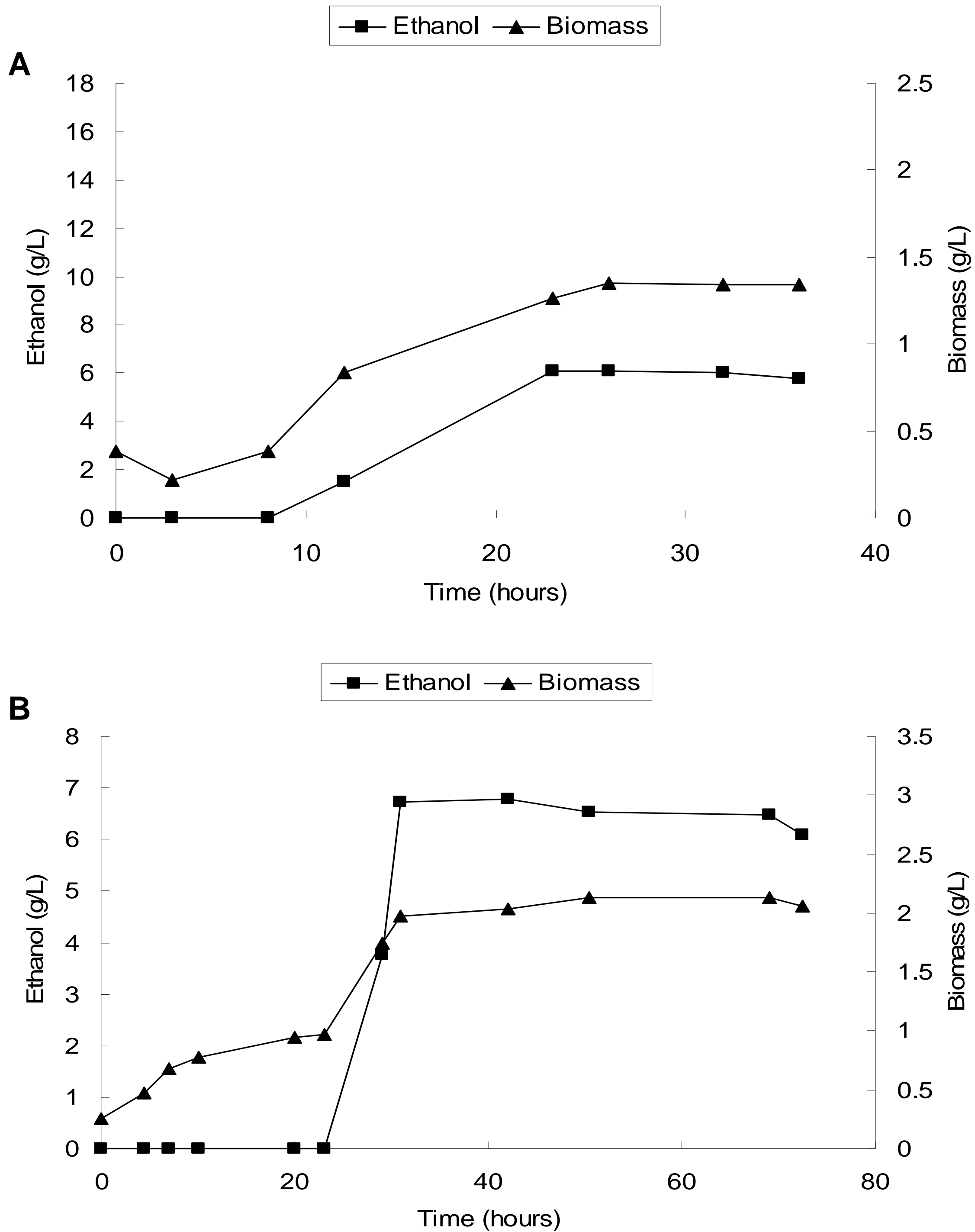


Fig. 4.13 Batch Fermentation with the Addition of 1 g/L of Glucoamylase. One hundred mL of pre-cultured NRRL Y-132 (A) or NRRL Y-132/pAMY (B) was used to inoculate the fermentor containing 2% soluble starch. Biomass changes and ethanol production over time were measured. Each of the batch fermentations was performed once.

ethanol and ~2.0 g/L of biomass were produced, and ethanol production was detectable after the first hour of fermentation (Figure 4.14A). The results are comparable to the fermentation performed on 2% glucose (Figure 4.9), suggesting that the availability of reducing sugars in the fermentor did affect cell proliferation, ethanol production rate and total ethanol yield, and low availability of utilizable reducing sugars in the fermentor delayed the production of ethanol and lowered the overall yield of cell mass and ethanol.

However, a parallel fermentation performed with NRRL Y-132/pAMY showed different results (Figure 4.14B). Over a 24-hour fermentation, 7.9 g/L of ethanol and 2.0 g/L of biomass were produced. Even though the availability of utilizable reducing sugars is the same for both batch fermentation experiments, ethanol production in the fermentation by NRRL Y-132/pAMY was delayed about 5 hours compared with the one by NRRL Y-132, and total ethanol yield (7.8 g/L) was lower than that of NRRL Y-132 (10.2 g/L). Prolonged incubation did not increase the overall ethanol yield. NRRL Y-132/pAMY showed a reduced ethanol production rate and overall ethanol yield compared to the wild type as shown in Figure 4.14, suggesting over-expression of barley α-amylase may have generated stress on yeast fermentation pathway.

Comparison between Figure 4.13A and Figure 4.14A shows that the addition of barley α-amylase (besides glucoamylase) did increase overall biomass and ethanol yield in NRRL Y-132; however, less effect was observed in NRRL Y-132/pAMY (Figure 4.13B and Figure 4.14B). In addition, the addition of barley α-amylase increased the initial rate of biomass and ethanol production in both NRRL Y-132 and NRRL Y-132/pAMY.

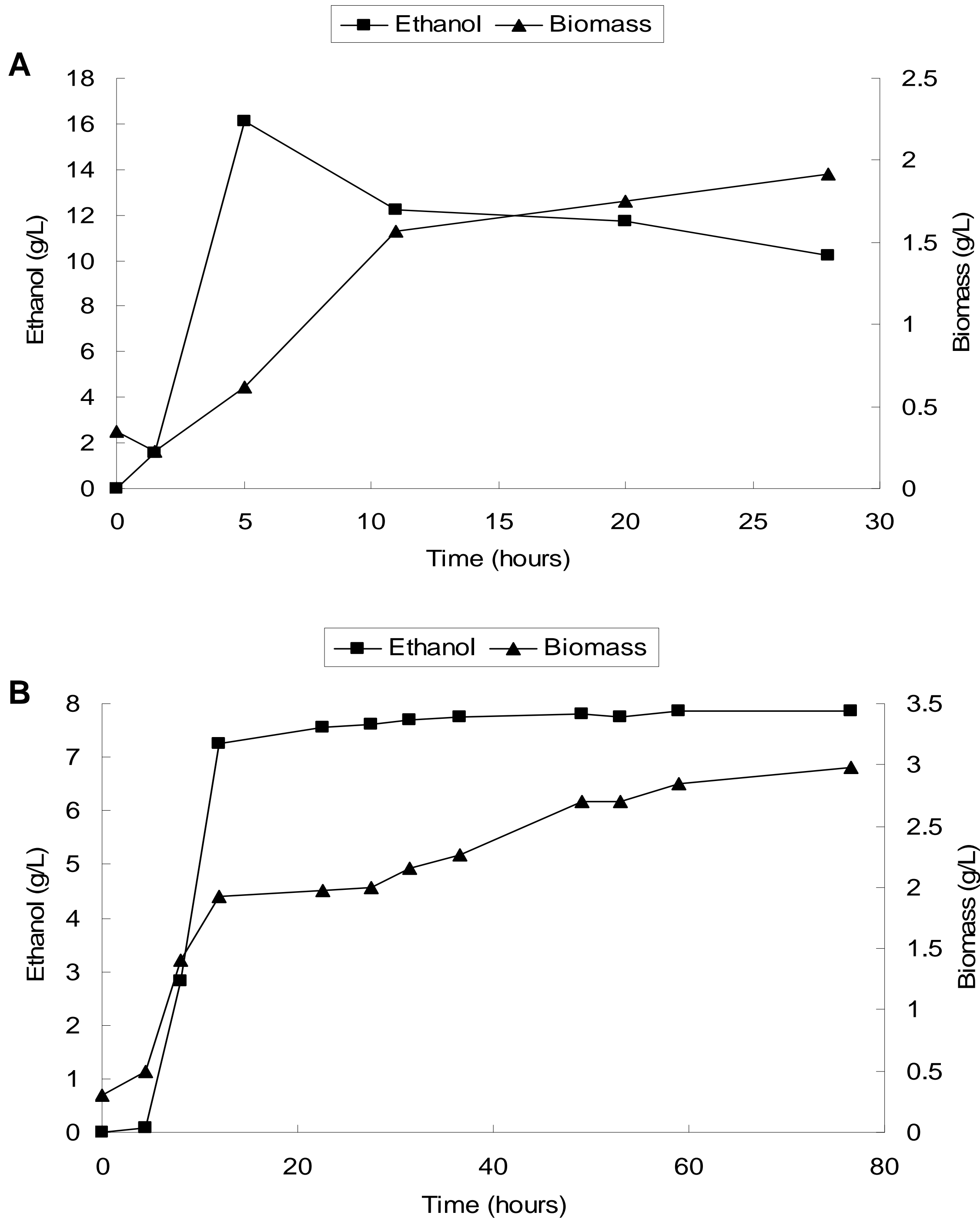
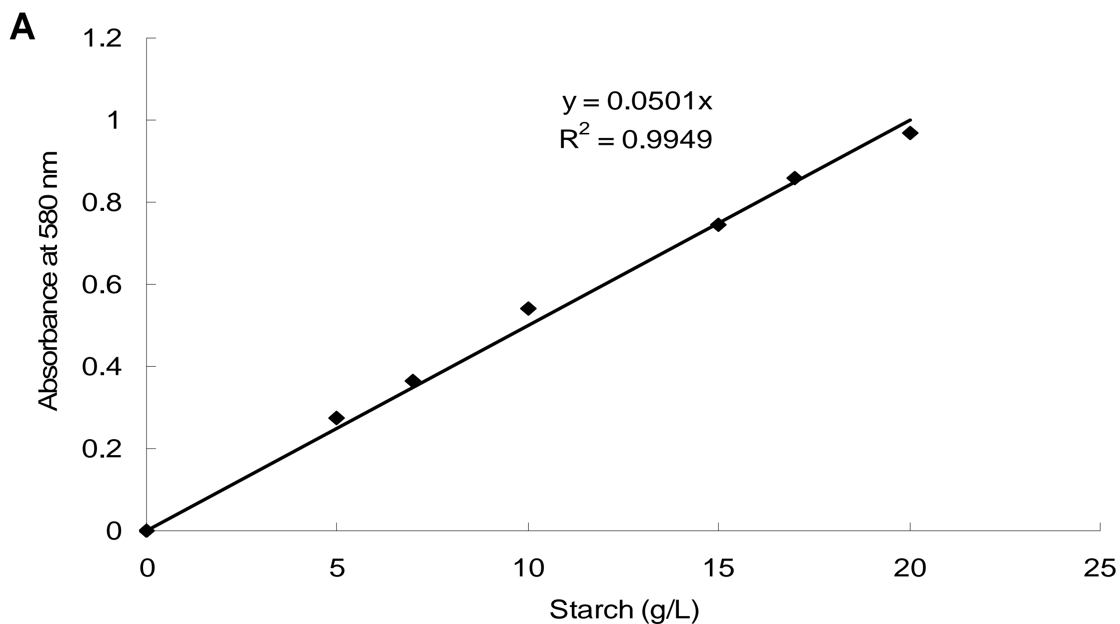


Fig. 4.14 Batch Fermentation with the Addition of 15 g/L of Barley α-Amylase and 1 g/L of Glucoamylase. One hundred mL of pre-cultured wild type NRRL Y-132 (A) or NRRL Y-132/pAMY (B) was used to inoculate the fermentor containing 2% soluble starch. Biomass changes and ethanol production over time were measured. Batch fermentations were performed once.

4.6.3 Measurement of Starch Concentration in Batch Fermentation Using Iodine Assay

Starch concentration is an important parameter to measure during batch fermentation. Since the Iodine assay was shown to be a valid method to measure starch concentration when quantifying α-amylase activity, the feasibility of using this assay to measure starch concentrations from samples collected from batch fermentation was investigated. Synthetic yeast media was used for all batch fermentations instead of YPD medium (section 3.2). It has been reported that the presence of YPD medium can cause a bleaching effect in the Iodine assay (Manonmani *et al.*, 1999). It was necessary to investigate whether the synthetic yeast media interfered with colour development in the Iodine assay. As detailed in section 3.6.7, known concentrations of soluble starch solutions were mixed with either water or synthetic yeast media before being analysed by the Iodine assay. Measured absorbances at 580 nm were plotted against the standard starch concentrations (Figure 4.15). The assay performed using water (panel A) generated similar results compared to the one using synthetic yeast medium (panel B), with both graphs showing a linear relationship between absorbances taken at 580 nm and standard starch concentrations. The results indicated that synthetic yeast media does not interfere with colour development in the Iodine assay. The graphs in Figure 4.15 were later used as standard curves for calculating starch concentrations in samples taken from the various batch fermentation runs.

When samples were frozen for future analysis, the starch became insoluble and formed aggregates upon being thawed at 37°C. Furthermore, yeast cells contained in the samples might also cause bleaching which could interfere with the Iodine assay. Because of these factors, samples were boiled in a water bath for 30 min before being analysed by the Iodine assay to re-solubilize the starch and break down yeast cells in the samples. For comparison, the same samples with or without boiling treatment were analysed by the Iodine assay. The results demonstrated that the measured starch concentrations were close to the expected values after boiling treatment (Table 4.3). In order to confirm that the measurements were accurate, and not affected by unknown factors present in the samples, known concentrations of soluble starch solutions were used to spike two other frozen samples collected at the end of a batch fermentation, which was performed with NRRL Y-132 on 2% soluble starch with the addition of 1 g/L of glucoamylase. The samples were boiled for 30 min and analysed by the Iodine



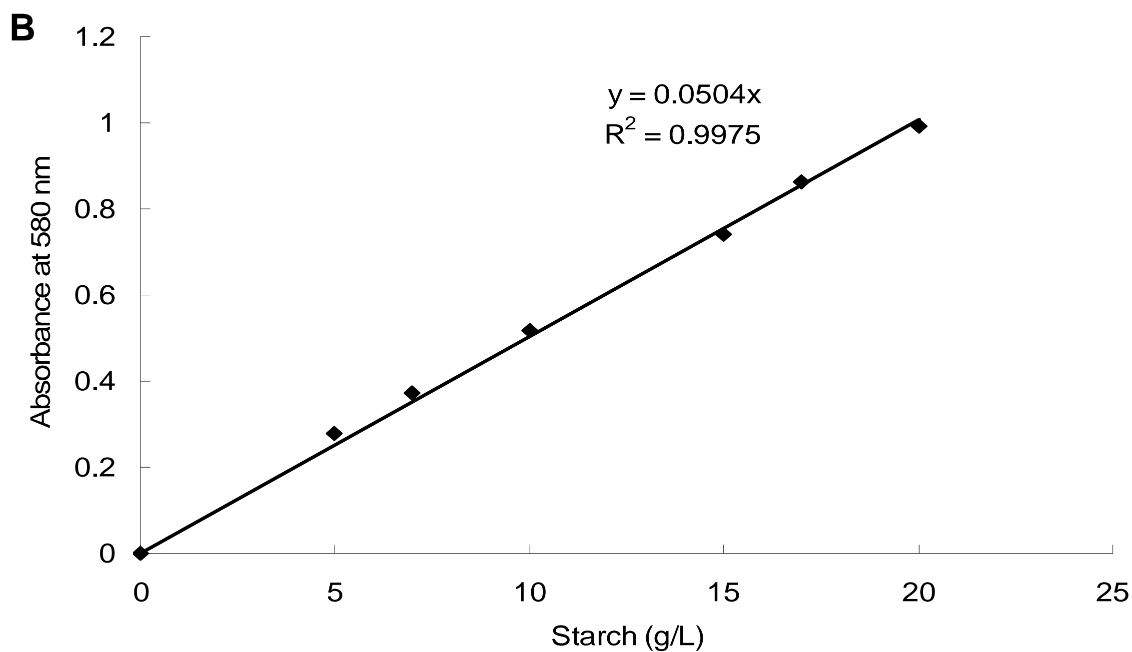


Fig. 4.15 Effect of Synthetic Yeast Media on the Iodine Assay When Measuring Starch Concentration. Solutions containing 100 µL of known concentrations of soluble starch and 100 µL of either water (A) or Synthetic yeast media (B) were subjected to the Iodine assay. The measured absorbances at 580 nm were plotted against standard starch concentrations (5, 7, 10, 15, 17, 20 g/L). The values are the averages of 3 independent experiments. The standard deviations were lower than 0.006 and therefore not shown.

TABLE 4.3 Effect of Boiling on Starch Concentration Determination in Frozen Samples

	Not Boiled		**Boiled**		
	Absorbance at 580 nm	[Starch] (g/L)	Absorbance at 580 nm	[Starch] (g/L)	Time (hours)
1	0.076	1.5	0.929	18.4	0
2	0.064	1.1	0.787	15.6	160

Samples were taken from a batch fermentation with wild type NRRL Y-132 on 2% soluble starch. Samples were stored at -20°C. Frozen samples were either boiled for 30 min or thawed at 37°C before being analysed. The two samples were taken at different time points as indicated above. Absorbance at 580 nm was measured and the readings were converted to starch concentration using the standard curve shown in Figure 4.15. The values are the averages of 2 independent experiments. The standard deviations were lower than 0.002 (not shown).

assay. Boiled samples were spiked with 100 μL of 20 g/L and 5 g/L soluble starch solutions, respectively, and analysed by Iodine assay (Table 4.4). The soluble starch concentrations in the spiked solutions were correctly measured by the Iodine assay. The results indicate that, with boiling treatment of the samples, the Iodine assay generates reliable measurements of starch concentration in samples collected from batch fermentations.

4.6.4 Plasmid Stability of NRRL Y-132 in Batch Fermentation

Plasmid stability in NRRL Y-132/pAMY is important for the maintenance of constitutive expression of cell surface anchored α-amylase. In small volume cultures of NRRL Y-132/pAMY, blasticidin was always added to ensure that all the cells maintained their plasmids. However, adding blasticidin is not practical for large-scale fermentation processes and may increase the difficulty of purifying the final product. The importance of blasticidin on maintaining the selection pressure on the amylolytic activity of NRRL Y-132/pAMY was investigated by performing the starch plate assay without addition of blasticidin. As shown in Figure 4.16A, no halo formation was observed around colonies in the absence of blasticidin. This indicated that NRRL Y-132/pAMY lost their amylolytic activity without the selection pressure of blasticidin.

As suggested in section 2.3.4, during batch fermentation, allowing NRRL Y-132/pAMY to grow on starch could act as a selection pressure since only yeast expressing α-amylase would be able to degrade starch and take up the released sugars for energy supply. To investigate whether the ability of yeast to grow on starch as the sole carbon source could act as a selection pressure, plasmid stability was measured in starch fermentations where NRRL Y-132/pAMY was used. As detailed in section 3.9, samples were taken from the fermentor at different time points, then diluted with sterile water. The same amount of diluted cell suspension was plated on two YPD plates and two other YPD plates containing 100 μg/mL of blasticidin. The number of colonies was counted from each plate after two days of incubation at 30°C, and plasmid stability was defined as the number of blasticidin resistant cells within the entire population of cells in the fermentor.

As described previously, three batch fermentations under different conditions were performed with NRRL Y-132/pAMY. In the batch fermentation with the addition of 1 g/L of glucoamylase (Figure 4.13B), the samples taken at the beginning of the fermentation (0 hour)

TABLE 4.4 Effect of Synthetic Yeast Media on Starch Concentration Determination

	Not spiked		Spiked		
	Absorbance at 580 nm	[Starch] (g/L)	Absorbance at 580 nm	[Starch] (g/L)	Time (hours)
1	0.008	0.0	0.952	18.9	72
2	-0.010	0.0	0.256	5.1	72

Samples were collected from batch fermentation performed with wild type NRRL Y-132 on 2% soluble starch with the addition of 1 g/L of glucoamylase. Frozen samples were boiled for 30 min and analysed by the Iodine assay (un-spiked). Then, known concentrations of starch solutions (**1**. 20 g/L; **2**. 5 g/L) were added to the boiled samples (spiked), respectively. Absorbance at 580 nm was measured for each sample and the readings were converted to starch concentration using the standard curve shown in Figure 4.15. The values are the averages of 2 independent experiments. The standard deviations were lower than 0.004 and therefore are not shown.

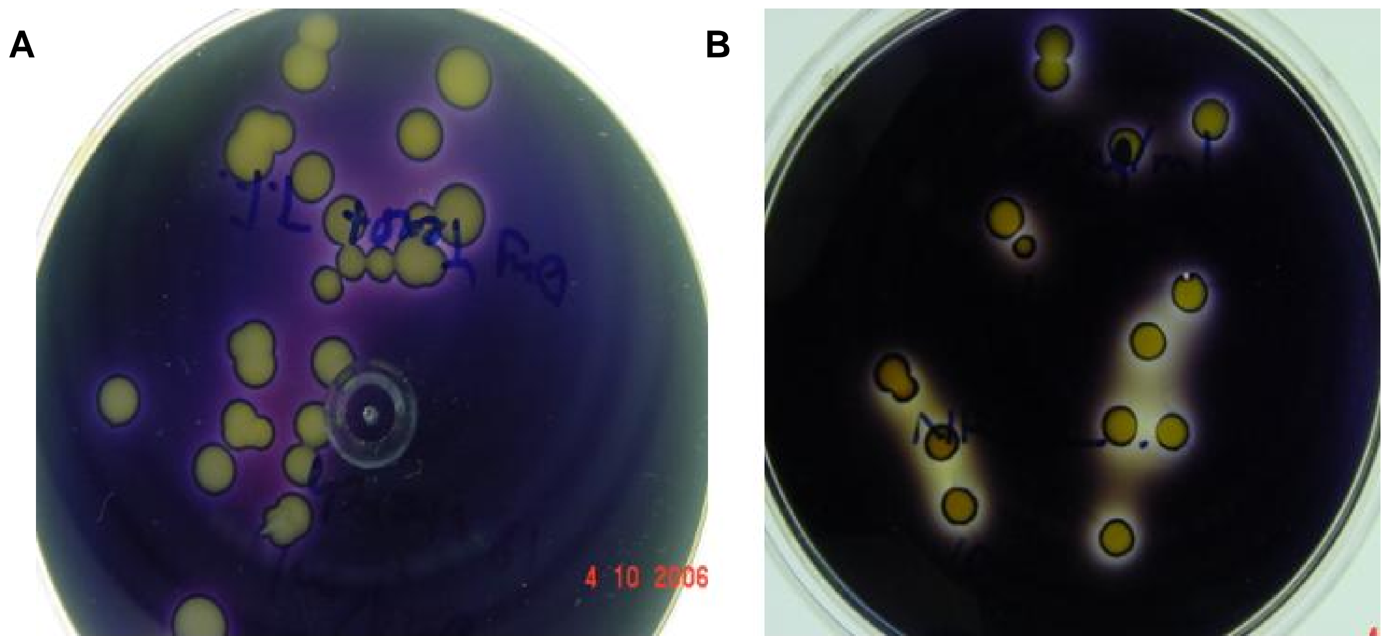


Fig. 4.16 Effect of Blasticidin Selection Pressure on Amylolytic Activity of NRRL Y-132/pAMY Using the Starch Plate Assay. A diluted cell suspension of NRRL Y-132/pAMY was plated on YPD plates containing 1% soluble starch without blasticidin (A) or with 100 μg/mL of blasticidin (B). Plates were incubated at 30°C for 2 days, then stained with iodine vapour.

showed that 100% of the yeast expressed the plasmid. After 23 hours, plasmid stability dropped slightly to 89%, and was maintained at this level until the end of the fermentation (Figure 4.17A). This suggested a much higher amount of plasmid-bearing cells in the fermentor than plasmid-free cells. When both barley α-amylase and glucoamylase were added into the fermentor, plasmid stability quickly dropped to 40% at the 12-hour point, and remained between 40-50% until the end of the fermentation (Figure 4.17B). In addition, compared with the plasmid stability described in the first fermentation experiment (Figure 4.17A), plasmid stability from this fermentation was much lower than the one with only glucoamylase added. For the batch fermentation without addition of any amylolytic enzymes, plasmid stability data was only available after 45 hours (Figure 4.18). At the 45-hour point, plasmid stability was approximately 12%. Following this, it gradually increased to 65% at the 116-hour point. Plasmid stability then remained between 75-85% until the end of the batch fermentation.

The last batch fermentation involved a prolonged fermentation period, and the plasmid stability remained relatively high until the end of the experiment (Figure 4.18A). For each sample point, the total number of colonies that grew on YPD plates was plotted against the number of colonies that grew on YPD plates with blasticidin. Interestingly, after 123.5 hours, the number of plasmid bearing cells remained at a relatively constant level. In contrast, it was the number of plasmid free cells that decreased over time (Figure 4.18B).

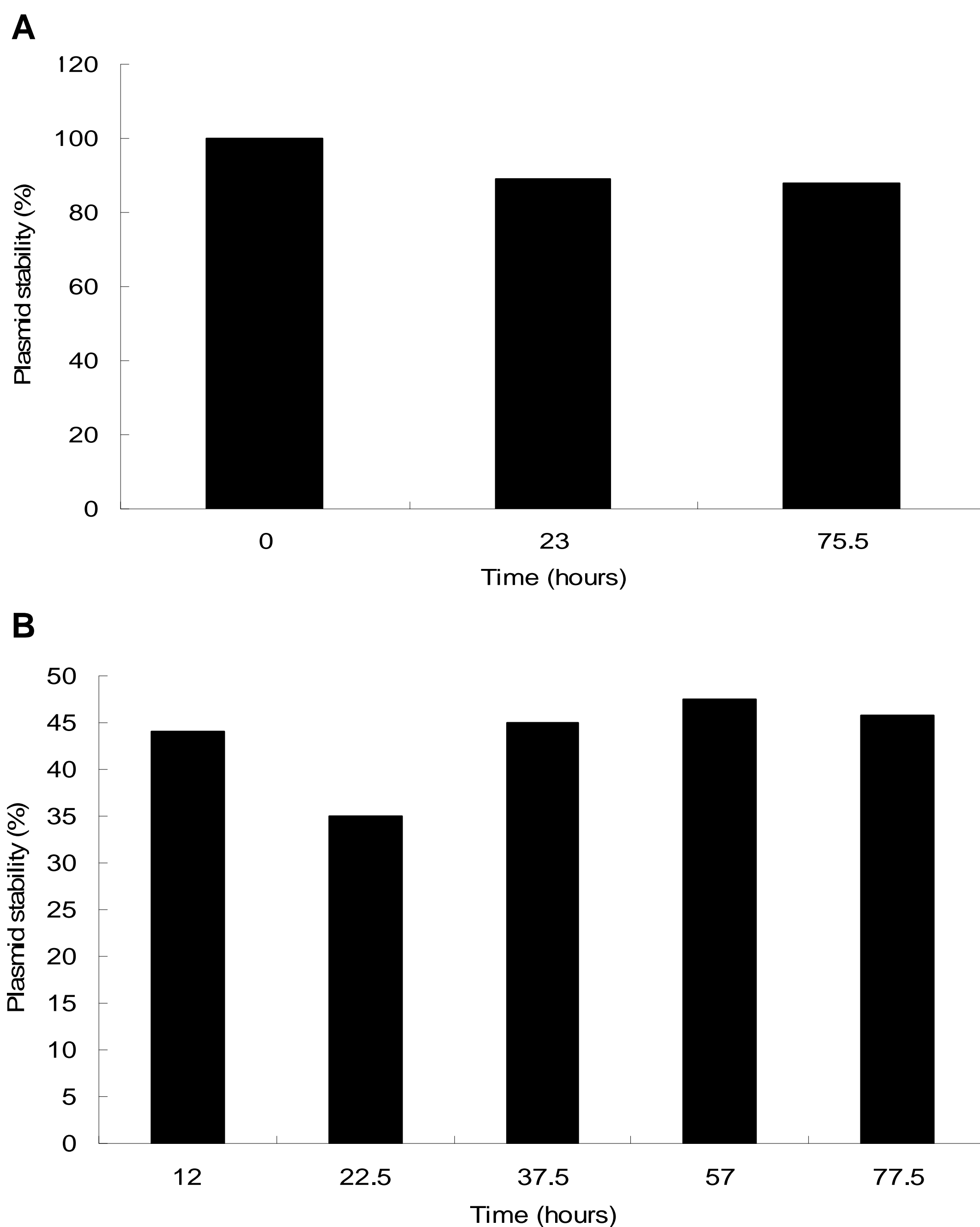
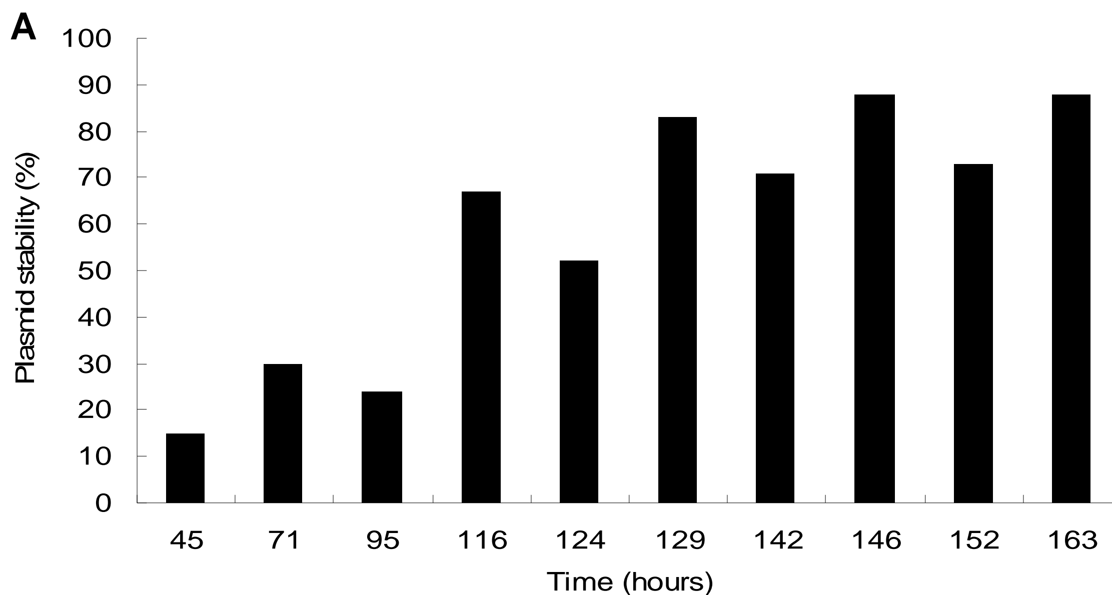


Fig. 4.17 Plasmid Stability During Batch Fermentation Using NRRL Y-132/pAMY with the Addition of Amylolytic Enzymes. (A) With addition of glucoamylase; (B) With addition of both glucoamylase and α-amylase. Samples were taken from the fermentor at the indicated time point as shown above. Samples were diluted with sterile water and the same amount of diluted cell suspension was plated on two YPD plates and two other YPD plates containing 100 μg/mL of blasticidin. Plasmid stability is shown as the percentage of colonies grown on blasticidin containing plates compared with colony number on plates without blasticidin.



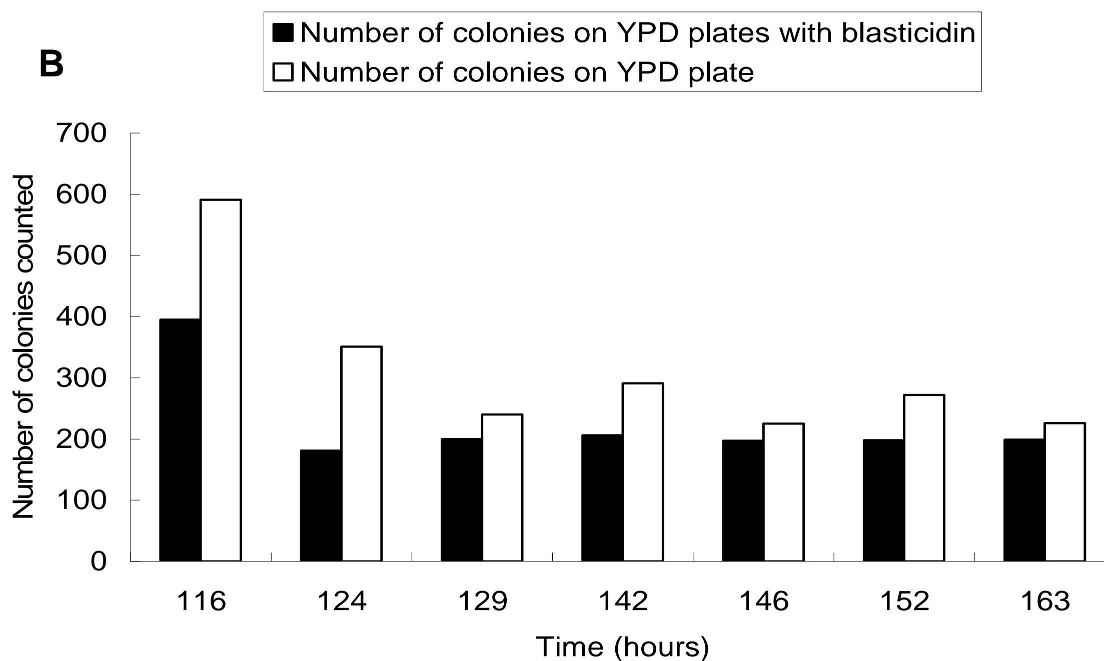


Fig. 4.18 Plasmid Stability During Batch Fermentation Using NRRL Y-132/pAMY without Addition of Amylolytic Enzymes. Samples were taken from the fermentor at the indicated time points. Samples were diluted with sterile water and the same amount of diluted cell suspension was plated on two YPD plates and two other YPD plates containing 100 μg/mL of blasticidin. Plasmid stability is shown as the percentage of the colonies grown on blasticidin containing plates compared with colony number on plates without blasticidin (A). The actual colony numbers counted from the plates with or without 100 μg/mL of blasticidin are also shown above (B).

5.0 DISCUSSION

5.1 Detection and Quantification of α-Amylase Activity in Intact Yeast

The cDNA encoding barley α-amylase was fused to a fragment encoding the 3' half of α-agglutinin, and the fusion gene was cloned downstream of the constitutive promoter *ADH1* in the yeast expression plasmid pAMY containing the *E. coli Amp* gene and the blasticidin resistance gene. Clones harbouring pAMY (NRRL Y-132/pAMY) were selected by incubating yeast cells on YPD agar plates containing 100 μg/mL of blasticidin. The amylolytic activity of the NRRL Y-132/pAMY clones was detected by using the starch plate assay, which generated visible haloes around the colonies out of the dark purple background on the YPD-starch plate after iodine vapour staining.

However, the starch plate assay does not generate quantitative data on α-amylase activity. Moraes *et al*. (1995) suggested that the size of the halo generated by the starch plate assay does not positively correspond to the amount of amylolytic activity displayed by a particular clone. Hence, the amylolytic activity generated by intact NRRL Y-132/pAMY was further quantified using the DNS and Iodine assays. Both assays were used to measure the starch-hydrolysing rate in the same reaction catalyzed by NRRL Y-132/pAMY. The DNS assay measured the amount of products, the reducing sugars, formed in the reaction solution, and the Iodine assay measured the amount of substrate, soluble starch, remaining in the reaction solution. The data showed that both assays directly assessed the reduction of substrate and accumulation of product in the 6-hour reaction, respectively, by observing changes in colour intensity. The results are consistent with the observation of the amylolytic activity generated by NRRL Y-132/pAMY using the starch plate assay, and confirmed the starch hydrolysis ability of NRRL Y-132/pAMY.

Interestingly, the specific activity of α-amylase measured by the Iodine assay was about 2 times higher than the one measured by the DNS assay (Table 4.1 and 4.2). Moreover, as shown in Figure 4.7 and 4.8, for the reactions catalyzed by NRRL Y-132/pAMY, after about 5 hours, no soluble starch was detected in the reaction solutions. However, reducing sugars were

measured at an almost linear rate until the end of the reaction. Assuming an equal mass conversion between starch and the reducing sugars generated from it, the amount of hydrolyzed starch measured by the Iodine assay should be equal to the amount of reducing sugars generated as is measured by the DNS assay. The reactions initiated in the presence of 5 mg/mL of soluble starch indicated the depletion of starch in the reaction solution after 4-5 hours. However, only 2.6 mg/mL of maltose were generated in the first 4 hours, and about a total of 4 mg/mL of reducing sugars was detected by the DNS assay after 6 hours. Surprisingly, the results appeared to suggest an unequal conversion rate between the substrate and the products. Based on the data, starch molecules were being broken down faster than the reducing sugars were generated.

Xiao *et al*. (2006) reported a similar inconsistency between the Iodine assay and DNS assay when quantifying the amylolytic activity of *Aspergillus oryzae* α-amylase. They found that the α-amylase activity measured with the Iodine assay was 5 times higher than the one measured by the DNS assay. They speculated that the inconsistency was mainly due to the nature of the amylolytic activity of α-amylase on starch molecules. α-Amylase hydrolyses starch molecules at the α-1,4 linkage, but only generates a small amount of reducing sugars. The oligosaccharides produced would not be detected by the Iodine assay. Thus, based on the results from the two assays, a higher starch hydrolysis rate was observed comparing to the reducing sugar generation rate. Xiao *et al*. (2006) also showed that better consistency was observed between the two assays when glucoamylase activity was measured, since glucoamylase removes one glucose residue from the non-reducing end of a starch polymer at a time. Collectively, the Iodine assay appears to more accurately measure α-amylase activity. However, the DNS assay generates more accurate information on the availability of fermentable sugars.

There are additional factors that may affect the assay results. Some yeast strains are able to utilize maltose or even maltotriose in addition to glucose. During batch fermentation studies, it was discovered that *S. cerevisiae* NRRL Y-132 could utilize maltose with high efficiency for cell proliferation and fermentation (Figure 4.10). This strain's ability to take up maltose may potentially affect the accuracy of the results from the DNS assay, since it is possible that the yeast cells take up some of the maltose generated from starch hydrolysis. Because of this, the amylolytic activity measured by the DNS assay could be potentially lower

than the actual α-amylase activity. The issue could be addressed in the future by performing a control experiment in which known concentrations of maltose are used in batch fermentation experiments, and the amount of maltose measured after certain periods of time to check the maltose uptake rate by NRRL Y-132/pAMY.

In addition, it has been reported that the Iodine assay is sensitive to temperature changes and the presence of reducing factors in the assay (Manonmani *et al*., 1999). The thiol groups present in most bacteria and yeast media could compete with starch polymers for iodine binding, and cause a bleaching effect on the assay. Because of this, the addition of both hydrogen peroxide and $CuSO_4 \cdot 5H_2O$ solutions into the assay was suggested to protect against a bleaching effect caused by reducing factors in the growth medium. However, during these assay experiments, it was found that both yeast cells and YPD medium cause a bleaching effect in the Iodine assay. When hydrogen peroxide and $CuSO_4 \cdot 5H_2O$ solutions were added into the solutions, it was difficult to obtain stable absorbances at 580 nm (data not shown). Alternatively, a different method was used to avoid these interferences. The YPD medium was removed by repeated washings with sodium acetate buffer (pH4.5), and yeast cells were separated from the samples by centrifugation (refer to section 3.5.2) before being subjected to the assay. In addition, since the starch-iodine complex is unstable at a temperature of 35°C or higher (data not shown), samples were first cooled on ice before they were used in the assay.

Even though YPD medium showed a strong bleaching effect in the Iodine assay, interestingly, synthetic yeast medium did not cause the bleaching effect, and gave the same result as when water was tested (Figure 4.15A). The results suggested the possibility of using the Iodine assay to measure starch concentration in batch fermentation samples (Table 4.3). As mentioned above, the results obtained by the Iodine assay may not be accurate for assaying reducing sugar formation. However, as long as the amount of utilizable reducing sugars in the fermentor is not an issue, the Iodine assay is an effective method to measure starch-hydrolysing rate in batch fermentation studies.

5.2 Effects of Cell Surface Anchoring on α-Amylase Activity

S. cerevisiae is an efficient expression system for heterologous genes. There are many examples of foreign proteins being expressed and secreted at high levels in yeast. Functional

amylolytic enzymes from various sources have been successfully expressed in yeast, and particularly, constitutive production of active barley α-amylase with kinetic properties comparable to barley seed α-amylase in yeast was reported (Wong *et al.*, 2002).

Even though it was shown in this work that NRRL Y-132/pAMY was able to hydrolyse soluble starch under conditions favourable for barley α-amylase activity (pH 4.5, 45°C) (Figure 4.7 and 4.8), the starch-hydrolysing rate of NRRL Y-132/pAMY was much lower than expected, and its starch hydrolysis rate was not sufficient to support yeast proliferation on soluble starch (Figure 4.10). The results suggested that the amylolytic activity catalysed by the intact NRRL Y-132/pAMY on soluble starch is reduced compared to that of the free enzyme, and possible causes are discussed below.

Reduction in activity has been observed for amylolytic enzymes expressed as fusion proteins or as cell surface anchored proteins, as detailed in section 2.4. The folding of a protein might be changed when it is expressed as part of a fusion protein, and this might lead to changes in its three dimensional structure and its catalytic activity. Also, when a protein is anchored on the cell wall, its motility towards substrates is reduced compared to free enzymes, and its accessibility to substrates may be hindered by the anchorage. Previous studies have shown that fusing the C-terminus of α-amylase to a glucoamylase resulted in a reduction or total loss of its catalytic activity (Moraes *et al*., 1995), and dramatic activity losses on α-amylase were observed when its C-terminus was anchored on the cell surface through α-agglutinin (Shigechi *et al*., 2004).

As stated in section 2.4.3, there is thought to be a substrate-binding site located within domain C, which was also reported to be a very flexible structure and may play an important role in facilitating the starch hydrolysis rate of barley α-amylase (Kadziola *et al*., 1998; Gottschalk *et al*., 2001; Robert *et al*., 2003, 2005). It is speculated that the anchoring process might make domain C a rather rigid structure (since it is directly fused with α-agglutinin), preventing the starch-binding site within this domain from having access to starch molecules. This could be further studied by inserting a flexible peptide linker between barley α-amylase and α-agglutinin. Changes in the binding constant (K_b) of cell wall anchored barley α-amylase can also be measured.

Assuming that the starch binding site on domain C was unable to gain access to its substrate due to the anchoring process, with both the catalytic site and another major starch-

binding site located at domain A, which is located at N-terminal of barley α-amylase and not directly affected by the anchoring process, barley α-amylase's activity should only be partially affected. However, this does not agree with my experimental observations: cell surface anchored barley α-amylase displayed only weak activity. Another possible explanation would be that these starch binding domains of barley α-amylase work in a cooperative manner rather than working independently from each other. It has been suggested that the starch-binding site at domain C may cooperate with the starch binding site at domain A to untangle and re-orient the α-helical structure of starch polymer so that the substrate is correctly positioned at the catalytic site (Robert *et al.*, 2003, 2005). However, there has been no direct evidence to support this hypothesis.

Furthermore, anchorage may not be the only reason behind the reduction in activity. The yeast cell wall structure may also interfere with the accessibility of cell surface anchored enzymes towards their substrates, especially long hydrophobic chain molecules such as starch. The outer layer of the yeast cell wall is covered with mannoprotein, and its hydrophobic nature may drive the hydrophobic chains of starch away. The barley α-amylase's accessibility towards starch molecules may be reduced if the enzyme is not further extended away from the cell surface.

5.3 Batch Fermentation on Soluble Starch

In industrial starch fermentation, it is not practical to add large amounts of antibiotics. Changes in plasmid stability were monitored in 3 different batch fermentations on 2% soluble starch using NRRL Y-132/pAMY. The observed plasmid stability varied greatly among the three batch fermentations. Comparing plasmid stability between the batch fermentation with only addition of *Rhyzopus* glucoamylase and the fermentation with addition of both barley α-amylase and *Rhyzopus* glucoamylase, plasmid stability in the former batch fermentation was almost twice as high than in the latter one (Figure 4.17). Starch hydrolysis was carried out at a faster rate when both α-amylase and glucoamylase were used relative to when glucoamylase was used during fermentation, so glucose availability was speculated to be a major factor causing the difference on plasmid stability between the two runs.

Based on two previous studies on plasmid stability using recombinant yeast in batch fermentation (Alintas *et al*., 2001; Kondo *et al*., 2002), in the absence of antibiotics, plasmid stability was directly affected by the amount of glucose available in the fermentor. It was reported that when there is a large amount of fermentable sugars available in the medium, plasmid-free cells tended to rapidly outgrow plasmid-bearing cells (Alintas *et al*., 2001). My experiment results are consistent with this report (Figure 4.17).

Interestingly, high plasmid stability was observed in batch fermentation using concentrated NRRL Y-132/pAMY as the inoculate (Figure 4.18A) even under prolonged incubation. In this case, starch potentially maintained selection pressure on the plasmid-bearing cells when the amount of glucose was limiting in the fermentor, since only plasmid-bearing cells were able to hydrolyse starch and release fermentable sugars that could be taken up by the cells. By limiting the amount of free glucose in the medium, the chances for plasmid-free cells and bacteria to proliferate are very small. However, the reason for low plasmid stability at the early stage of fermentation is unclear. It is possible that the fluctuation that occurred in the numbers of plasmid free cells may have been due to the utilization of nutrients from dead cells in the fermentor (Figure 4.18B). There was a large biomass drop at the 60-hour point (Figure 4.10B). Cells may have died at that point due to a lack of glucose caused by a lack of sufficient amylolytic activity.

Inhibition of enzyme activity caused by accumulation of products in the fermentation medium can be an issue when soluble amylolytic enzymes or those secreted by recombinant yeast are used for starch hydrolysis. During my project, I obtained no actual data that suggested that product inhibition was occurring. Kondo *et al*. (2002) anchored *Rhyzopus oryzae* glucoamylase on yeast cell surface. In an 80 hours starch fermentation utilizing this genetically modified yeast strain, glucose could barely be detected in the fermentation medium. They suggested that since all of the amylolytic enzymes were anchored on the cell surface, starch hydrolysis was occurring in close proximity to the recombinant cells. Because of this particular circumstance, almost all of the glucose released from starch hydrolysis would be readily taken up by the yeast cells and no accumulation of glucose would be found in the fermentation medium. Based on their data, even though there is no direct evidence for product inhibition during cell surface anchored amylolytic experiments, it can be speculated that the glucose concentration close to the yeast cell surface was much higher than in the rest of the

medium. I speculate that the uptake of glucose would be very quick and product inhibition was not a major issue for cell surface anchored amylolytic enzyme activity during starch fermentation.

5.4 Conclusions

Various biotechniques have been used to improve industrial ethanol production by fermentation. Two different approaches have been generally used to achieve this goal. One approach is to engineer amylolytic enzymes to optimize their functionality during starch hydrolysis. The other approach is to select microorganisms with superior cellular abilities for ethanol productivity by mutation or metabolic engineering. In this work, the two approaches were combined: barley α-amylase was engineered to be immobilized on the yeast cell wall and the new recombinant yeast gained starch utilizing ability. The barley α-amylase gene was fused with a cell surface-anchoring gene, α-agglutinin, and inserted into a yeast expression plasmid. An antibiotic resistance gene, blasticidin, was inserted into the expression plasmid as the selection marker, so that this new plasmid can be generally used to engineer a broad range of wild type industrial ethanol producing strains rather than the genetically modified yeast strains only available from research laboratories.

To quantify expression, DNS and Iodine assays that are generally used to quantify amylolytic enzyme activities were further developed in this work. Under specific assay conditions (pH4.5, 45°C), 100 mL NRRL Y-132/pAMY cell suspension was able to hydrolyse 5 mg/mL of soluble starch in 6 hours.

The ability of NRRL Y-132 and NRRL Y-132/pAMY on starch utilization and ethanol production was studied by anaerobic, batch fermentations using soluble starch as the sole carbon source. The results of batch fermentation study demonstrated that NRRL Y-132 is a maltose utilizing strain, and glucoamylase activity may not be required for this particular strain during starch fermentation (Figure 4.12). Strain NRRL Y-132/pAMY was able to show starch-utilizing ability under the fermentation conditions with soluble starch as the sole carbon source. However, due to low amylolytic activity on the intact cell surface, the biomass yield was much lower than when glucose was used as the carbon source, and no ethanol was generated during the starch fermentation. It is hypothesized that an improved cell surface amylolytic activity can

be achieved by employing an alternative anchoring protein, which will be further discussed in section 5.5.

In addition, to study the effects of over-expression of cell surface anchored barley α-amylase on cell metabolism, the overall biomass yield and ethanol producing ability of NRRL Y-132/pAMY were compared with those of NRRL Y-132 under the same batch fermentation conditions. Even though NRRL Y-132/pAMY showed a lower biomass generation rate and a delay in ethanol production, the overall ethanol production was at a similar level as NRRL Y-132.

Furthermore, without the addition of antibiotics, high plasmid stability was observed during starch fermentations utilizing NRRL Y-132/pAMY. This is probably due to the starch utilizing ability that acted as a positive selection pressure. This suggests that this approach can be used to maintain high plasmid stability during industrial fermentation processes without the need for supplying costly and contaminating antibiotics.

5.5 Future Directions

The anchoring of barley α-amylase on yeast cell surface with α-agglutinin might have reduced the accessibility of starch molecules to the starch-binding site at its C-terminus. Thus, a different anchoring protein such as flocculation protein 1 should be tested. As detailed in section 2.3.2.3, flocculation protein 1 is also a cell surface protein, which is involved in the flocculation ability in certain yeast strains. Structural studies have shown that flocculation protein 1 has two distinct domains that have cell wall anchoring properties: GPI anchoring domain at its C-terminus and mannoprotein binding domain at its N-terminus. It has been reported that the N-terminal mannoprotein binding domain could bind non-covalently to the mannoproteins on the outer layer of yeast cell wall (Takeshi *et al*., 2002). By deleting C-terminal GPI anchoring domain of flocculation protein 1, its N-terminal mannoprotein binding domain can be used to anchor barley α-amylase on yeast cell wall. In this way, barley α-amylase is anchored on cell wall through its N-terminus with its C-terminal starch binding domain having free access to substrates (Figure 4.19). To date, a *Rhizopus oryzae* lipase (Takeshi. *et al*., 2002) and a bacteria α-amylase (Shigechi *et al*., 2004) were successfully anchored on the cell surface by using flocculation protein 1. Importantly, the studies were able

to show that the two enzymes, after being anchored by flocculation protein 1, showed 60 times higher activity than when they were anchored by α-agglutinin.

Alternatively, based on the current design, a peptide linker might be inserted between barley α-amylase and α-agglutinin. Suitable peptide linkers with different lengths could be studied. The peptide linkers may restore the flexibility of domain C of barley α-amylase and also extend the enzyme further away from the cell wall structure to increase its ability to bind to the starch substrate.

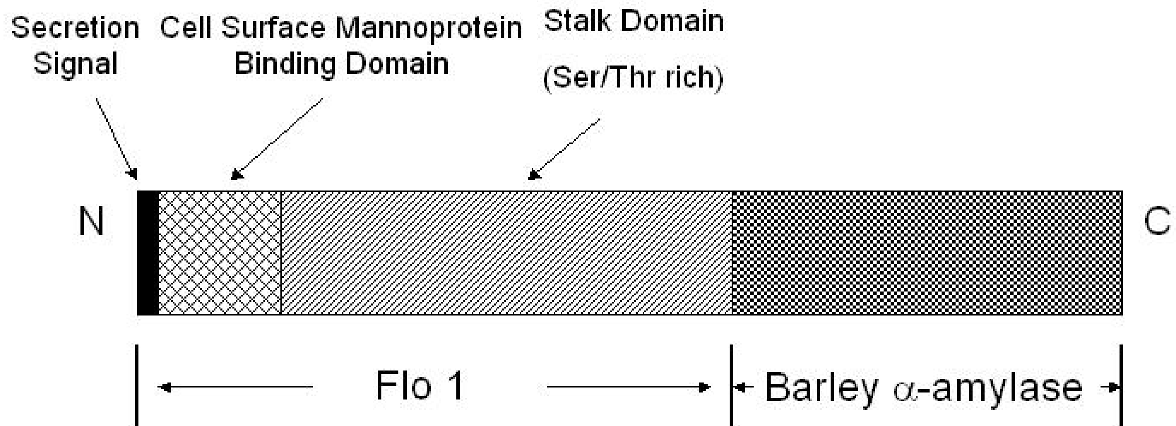


Figure 4.19 Structure of Fusion Protein Containing Flocculation Protein 1 as the Anchoring Protein. The C-terminal GPI anchoring signal was removed from flocculation protein 1 in this design so that its mannoprotein binding domain will be responsible for cell wall anchorage. Barley α-amylase will be extended away from cell wall structure by the stalk domain of flocculation protein 1.

6.0 REFERENCES

Altintas, M. M., Kirdar, B., Onsan, Z. and Ulgen, K. O. (2001) Plasmid stability in a recombinant *Saccharomyces cerevisiae* strain secreting a bifunctional fusion protein. *J. Chem. Technol. Biotechnol.* **76,** 612-618.

Aristidou, A. and Penttila, M. (2000) Metabolic engineering applications to renewable resource utilization. *Curr. Opin. Biotechnol.* **11,** 187-198.

Ashikari, T., Kiuchi-Goto, N., Tanaka, Y., Shibano, Y., Amachi, T. and Yoshizumi, H. (1989) High expression and efficient secretion of *Rhizopus oryzae* glucoamylase in the yeast *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **30,** 515-520.

Ashikari, T., Nakamura, N., Tanaka, Y., Kiuchi, N., Shibano, Y., Tanaka, T., Amachi, T. and Yoshizumi, H. (1986) *Rhizopus* Raw-starch-degrading glucoamylase: its cloning and expression in yeast. *Agri. Biol. Chem.* **50,** 957-964.

Ashikari, T., Nakamura, N., Tanaka, Y., Kiuchi, N., Shibano, Y., Tanaka, T., Amachi, T. and Yoshizumi, H. (1985) Cloning and expression of the *Rhizopus* glucoamylase gene in yeast. *Agri. Biol. Chem.* **49,** 2521-2523.

Birol, G., Onsan, Z., Kirdar, B. and Oliver, S. G. (1998) Ethanol production and fermentation characteristics of recombinant *Saccharomyces cerevisiae* strains grown on starch. *Enzyme Microb. Technol.* **22,** 672-677.

Bitter, G. A. and Egan, K. M. (1984) Expression of heterologous genes in *Saccharomyces cerevisiae* from vectors utilizing the glyceraldehyde-3-phosphate dehydrogenase gene promoter. *Gene* **32,** 263-274.

Bony, M., Thines-Sempoux, D., Barre, P. and Blondin, B. (1997) Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. *J. Bacteriol.* **179,** 4929-4936.

Bothast, R. J. and Schlicher, M. A. (2005) Biotechnological process for conversion of corn into ethanol. *Appl. Microbiol. Biotechnol.* **67,** 19-25.

Bourne, J. K., Jr. (2007) Biofuel:boon or boondoggle? *Nat. Geogr.* Oct, 38-59.

Bozonnet, S., Jensen, M. T., Nielsen, M. M., Aghajari, N., Jensen, M. H., Kramhoft, B., Willemoes, M., Tranier, S., Haser, R. and Svensson, B. (2007) The 'pair of sugar tongs' site on the non-catalytic domain C of barley α-amylase participates in substrate binding and activity. *FEBS Lett.* **274,** 5055-5067.

Cappellaro, C., Baldermann, C., Rachel, R. and Tanner, W. (1994) Mating type-specific cell-cell recognition of *Saccharomyces cerevisiae*: cell wall attachment and active sites of a- and α-agglutinin. *EMBO J.* **13,** 4737-4744.

Cappellaro, C., Hauser, K., Mrsa, V., Watzele, M., Watzele, G., Gruber, C. and Tanner, W. (1991) *Saccharomyces cerevisiae* a- and α-agglutinin: characterization of their molecular interaction. *EMBO J.* **10,** 4081-4088.

Chen, M. H., Shen, Z. M., Bobin, S., Kahn, P. C. and Lipke, P. N. (1995) Structure of *Saccharomyces cerevisiae* α-agglutinin. Evidence for a yeast cell wall protein with multiple immunoglobulin-like domains with atypical disulfides. *J. Biol. Chem.* **270,** 26168-26177.

de Moraes, L. M., Astolfi-Filho, S. and Oliver, S. G. (1995) Development of yeast strains for the efficient utilisation of starch: evaluation of constructs that express α-amylase and glucoamylase separately or as bifunctional fusion proteins. *Appl. Microbiol. Biotechnol.* **43,** 1067-1076.

Editorial. (2006) Bioethanol needs biotech now. *Nat. Biotech.* **24,** 725.

Farrell, A. E., Plevin, R. J., Turner, B. T., Jones, A. D., O'Hare, M. and Kammen, D. M. (2006) Ethanol can contribute to energy and environmental goals. *Science* **311,** 506-508.

Filho, S. A., Galembeck, E. V., Faria, J. B. and Frascino, A. C. S. (1986) Stable yeast transformants that secrete functional α-amylase encoded by cloned mouse pancreatic cDNA. *Nat. Biotech.* **4,** 311-315.

Fujita, Y., Ito, J., Ueda, M., Fukuda, H. and Kondo, A. (2004) Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. *Appl. Environ. Microbiol.* **70,** 1207-1212.

Geiger, J. H. (2003) Sugar tongs get a grip on the starch granule in barley α-amylase 1. *Structure* **11,** 903-904.

Gottschalk, T. E., Tull, D., Aghajari, N., Haser, R. and Svensson, B. (2001) Specificity modulation of barley α-amylase through biased random mutagenesis involving a conserved tripeptide in beta --> α loop 7 of the catalytic (β/α)(8)-barrel domain. *Biochemistry* **40,** 12844-12854.

Hill, G. A., Macdonald, D. G. and Lang, X. (1997) α-Amylase inhibition and inactivation in barley malt during cold starch hydrolysis. *Biotech. Lett.* **19,** 1139-1141.

Himmel, M. E., Ding, S., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W. and Foust, T. D. (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* **315,** 804-807.

Ho, N. W. Y., Chen, Z., Brainard, A. P. and Sedlak, M. (1999) Successful design and development of genetically engineered *Saccharomyces* yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to fuel ethanol. *Adv. Biochem. Eng.* **65,** 163-192.

Huang, G., Zhang, M. and Erdman, S. E. (2003) Posttranslational modifications required for cell surface localization and function of the fungal adhesin Aga1p. *Euk. Cell* **2,** 1099-1114.

Ingeldew, W. M. (1998) Alcohol production by *Saccharomyces cerevisiae*: a yeast primer; in *The Alcohol Textbook,* Jacques, K. A., Lyons, T. P. and Kelsali, D. R. (eds.), pp. 49-83, Nottingham University Press, Nottingham, UK.

Ingledew, W. M. (1993) Yeasts for production of fuel ethanol; in *The Yeasts,* Rose, A. H. and Harrison, J. S. (eds.), pp. 245-291, Academic Press, New York.

Izumi, M., Yamaguchi, I., Kobayashi, K., Endo, T., and Hanaoka, F. (1991) The blasticidin S resistance gene (bsr): a novel selectable marker for mammalian cells. *Exp. Cell Res.* **197,** 229-233.

Janse, B. J., Steyn, A. J. and Pretorius, I. S. (1993) Regional sequence homologies in starch-degrading enzymes. *Curr. Genet.* **24,** 400-407.

Juge, N., Nohr, J., Le Gal-Coeffet, M. F., Kramhoft, B., Furniss, C. S., Planchot, V., Archer, D. B., Williamson, G. and Svensson, B. (2006) The activity of barley α-amylase on starch granules is enhanced by fusion of a starch binding domain from *Aspergillus niger* glucoamylase. *Biochim. Biophys. Acta* **1764,** 275-284.

Juge, N., Sogaard, M., Chaix, J. C., Martin-Eauclaire, M. F., Svensson, B., Marchis-Mouren, G. and Guo, X. J. (1993) Comparison of barley malt α-amylase isozymes 1 and 2: construction of cDNA hybrids by in vivo recombination and their expression in yeast. *Gene* **130,** 159-166.

Kadziola, A., Abe, J., Svensson, B. and Haser, R. (1994) Crystal and molecular structure of barley α-amylase. *J. Mol. Biol.* **239,** 104-121.

Kadziola, A., Sogaard, M., Svensson, B. and Haser, R. (1998) Molecular structure of a barley α-amylase-inhibitor complex: implications for starch binding and catalysis. *J. Mol. Biol.* **278,** 205-217.

Katahira, S., Fujita, Y., Mizuike, A., Fukuda, H. and Kondo, A. (2004) Construction of a xylan-fermenting yeast strain through codisplay of xylanolytic enzymes on the surface of xylose-utilizing Saccharomyces cerevisiae cells. *Appl. Environ. Microbiol.* **70,** 5407-5414.

Khursheed, B. and Rogers, J. C. (1988) Barley α-amylase genes. Quantitative comparison of steady-state mRNA levels from individual members of the two different families expressed in aleurone cells. *J. Biol. Chem.* **263,** 18953-18960.

Kondo, A., Shigechi, H., Abe, M., Uyama, K., Matsumoto, T., Takahashi, S., Ueda, M., Tanaka, A., Kishimoto, M. and Fukuda, H. (2002) High-level ethanol production from starch by a flocculent *Saccharomyces cerevisiae* strain displaying cell-surface glucoamylase. *Appl. Microbiol. Biotechnol.* **58,** 291-296.

Lang, X., Hill, G. A. and Macdonald, D. G. (2001) Recycle bioreactor for bioethanol production from wheat starch I. Cold enzyme hydrolysis. *Energy Sources* **23,** 417-425.

Lang, X., Macdonald, D. G. and Hill, G. A. (2001) Recycle bioreactor for bioethanol production from wheat starch II. Fermentation and economics. *Energy Sources* **23,** 427-436.

Lim, L. H., Macdonald, D. G. and Hill, G. A. (2003) Hydrolysis of starch particles using immobilized barley α-amylase. *Biochem. Eng. J.* **13,** 53-62.

Lipke, P. N. and Kurjan, J. (1992) Sexual agglutination in budding yeasts: structure, function, and regulation of adhesion glycoproteins. *Microbiol. Rev.* **56,** 180-194.

Lipke, P. N. and Ovalle, R. (1998) Cell wall architecture in yeast: new structure and new challenges. *J. Bacteriol.* **180,** 3735-3740.

Lipke, P. N., Wojciechowicz, D. and Kurjan, J. (1989) AGα 1 is the structural gene for the *Saccharomyces cerevisiae* α-agglutinin, a cell surface glycoprotein involved in cell-cell interactions during mating. *Mol. Cell. Biol.* **9,** 3155-3165.

Lu, C. F., Montijn, R. C., Brown, J. L., Klis, F., Kurjan, J., Bussey, H. and Lipke, P. N. (1995) Glycosyl phosphatidylinositol-dependent cross-linking of α-agglutinin and beta 1,6-glucan in the *Saccharomyces cerevisiae* cell wall. *J. Cell Biol.* **128,** 333-340.

Lynd, L. R., van Zyl, W. H., McBride, J. E. and Laser, M. (2005) Consolidation bioprocessing of cellulosic biomasss: an update. *Curr. Opin. Biotechnol* **16,** 577-583.

Manonmani, H. K. and Kunhi, A. A. M. (1999) Interference of thiol-compounds with dextrinizing activity assay of α-amylase by starch-iodine colour reaction: modification of the method to eliminate this interference. *World J. Microbiol. Biotechnol.* **15,** 485-487.

Matsumoto, T., Fukuda, H., Ueda, M., Tanaka, A. and Kondo, A. (2002) Construction of yeast strains with high cell surface lipase activity by using novel display systems based on the Flo1p flocculation functional domain. *Appl. Environ. Microbiol.* **68,** 4517-4522.

Miki, B. L., Poon, N. H., James, A. P. and Seligy, V. L. (1982) Possible mechanism for flocculation interactions governed by gene FLO1 in *Saccharomyces cerevisiae*. *J. Bacteriol.* **150,** 878-889.

Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31,** 426-428.

Murai, T., Ueda, M., Atomi, H., Shibasaki, Y., Kamasawa, N., Osumi, M., Kawaguchi, T., Arai, M. and Tanaka, A. (1997) Genetic immobilization of cellulase on the cell surface of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **48,** 499-503.

Murai, T., Ueda, M., Shibasaki, Y., Kamasawa, N., Osumi, M., Imanaka, T. and Tanaka, A. (1999) Development of an arming yeast strain for efficient utilization of starch by co-display of sequential amylolytic enzymes on the cell surface. *Appl. Microbiol. Biotechnol.* **51,** 65-70.

Murai, T., Ueda, M., Yamamura, M., Atomi, H., Shibasaki, Y., Kamasawa, N., Osumi, M., Amachi, T. and Tanaka, A. (1997) Construction of a starch-utilizing yeast by cell surface engineering. *Appl. Environ. Microbiol.* **63,** 1362-1366.

Nakamura, Y., Kobayashi, F., Ohnaga, M. and Sawada, T. (1996) Alcohol fermentation of starch by a genetic recombinant yeast having glucoamylase activity. *Biotechnol. Bioeng.* **53,** 21-25.

Nakamura, Y., Sawada, T. and Komatsu, A. (2002) Ethanol production from raw starch by a recombinant yeast having saccharification and fermentation activities. *J. Chem. Technol. Biotechnol.* **77,** 1101-1106.

Nielsen, P. K., Bonsager, B. C., Fukuda, K. and Svensson, B. (2004) Barley α-amylase/subtilisin inhibitor: structure, biophysics and protein engineering. *Biochim. Biophys. Acta.* **1696,** 157-164.

Ophardt, C. E. (2003) *Virtual chembook*. URL(http://www.elmhurst.edu/~chm/vchembook/)

Robert, X., Haser, R., Gottschalk, T. E., Ratajczak, F., Driguez, H., Svensson, B. and Aghajari, N. (2003) The structure of barley α-amylase isozyme 1 reveals a novel role of domain C in substrate recognition and binding: a pair of sugar tongs. *Structure* **11,** 973-984.

Robert, X., Haser, R., Mori, H., Svensson, B. and Aghajari, N. (2005) Oligosaccharide binding to barley α-amylase 1. *J. Biol. Chem.* **280,** 32968-32978.

Rogers, J. C. (1985) Two barley α-amylase gene families are regulated differently in aleurone cells. *J. Biol. Chem.* **260,** 3731-3738.

Rogers, J. C. and Milliman, C. (1984) Coordinate increase in major transcripts from the high pI α-amylase multigene family in barley aleurone cells stimulated with gibberellic acid. *J. Biol. Chem.* **259,** 12234-12240.

Rogers, J. C. and Milliman, C. (1983) Isolation and sequence analysis of a barley α-amylase cDNA clone. *J. Biol. Chem.* **258,** 8169-8174.

Rothstein, S. J., Lazarus, C. M., Smith, W. E., Baulcombe, D. C. and Gatenby, A. A. (1984) Secretion of a wheat α-amylase expressed in yeast. *Nature* **308,** 662-665.

Roy, A., Lu, C. F., Marykwas, D. L., Lipke, P. N. and Kurjan, J. (1991) The *AGA1* product is involved in cell surface attachment of the *Saccharomyces cerevisiae* cell adhesion glycoprotein a-agglutinin. *Mol. Cell. Biol.* **11,** 4196-4206.

Ruohonen, L., Aalto, M. K. and Keranen, S. (1995) Modifications to the *ADH1* promoter of *Saccharomyces cerevisiae* for efficient production of heterologous proteins. *J. Biotechnol.* **39,** 193-203.

Ruohonen, L., Hackman, P., Lehtovaara, P., Knowles, J. K. C. and Keranen, S. (1987) Efficient secretion of *Bacillus amyloliquefaciens* α-amylase cells by its own signal peptide from *Saccharomyces cerevisiae* host. *Gene* **59,** 161-170.

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd (edn) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Schreuder, M. P., Brekelmans, S., van den Ende, H. and Klis, F. M. (1993) Targeting of a heterologous protein to the cell wall of *Saccharomyces cerevisiae*. *Yeast* **9,** 399-409.

Schreuder, M. P., Mooren, A. T., Toschka, H. Y., Verrips, C. T. and Klis, F. M. (1996) Immobilizing proteins on the surface of yeast cells. *Trends Biotechnol.* **14,** 115-120.

Service, R. F. (2007) Biofuel researchers prepare to reap a new harvest. *Science* **315,** 1488-1491.

Shen, Z. M., Wang, L., Pike, J., Jue, C. K., Zhao, H., de Nobel, H., Kurjan, J. and Lipke, P. N. (2001) Delineation of functional regions within the subunits of the *Saccharomyces cerevisiae* cell adhesion molecule a-agglutinin. *J. Biol. Chem.* **276,** 15768-15775.

Shigechi, H., Koh, J., Fujita, Y., Matsumoto, T., Bito, Y., Ueda, M., Satoh, E., Fukuda, H. and Kondo, A. (2004) Direct production of ethanol from raw corn starch via fermentation by use of a novel surface-engineered yeast strain codisplaying glucoamylase and α-amylase. *Appl. Environ. Microbiol.* **70,** 5037-5040.

Sogaard, M., Olsen, F. L. and Svensson, B. (1991) C-terminal processing of barley α-amylase 1 in malt, aleurone protoplasts, and yeast. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 8140-8144.

Southgate, V. J., Steyn, A. J., Pretorius, I. S. and Van Vuuren, H. J. (1993) Expression and secretion of *Bacillus amyloliquefaciens* α-amylase by using the yeast pheromone α-factor promoter and leader sequence in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **59,** 1253-1258.

Steyn, A. J. and Pretorius, I. S. (1991) Co-expression of a *Saccharomyces diastaticus* glucoamylase-encoding gene and a *Bacillus amyloliquefaciens* α-amylase-encoding gene in *Saccharomyces cerevisiae*. *Gene* **100,** 85-93.

Straver, M. H., Smit, G. and Kijne, J. W. (1994) Purification and partial characterization of a flocculin from brewer's yeast. *Appl. Environ. Microbiol.* **60,** 2754-2758.

Sumitani, J., Tottori, T., Kawaguchi, T. and Arai, M. (2000) New type of starch-binding domain: the direct repeat motif in the C-terminal region of *Bacillus sp.* no. 195 α-amylase contributes to starch binding and raw starch degrading. *Biochem. J.* **350 Pt 2,** 477-484.

Textor, S. D., Hill, G. A., Macdonald, D. G. and Denis, E. (1998) Cold enzyme hydrolysis of wheat starch granules. *Can. J. Chem. Eng.* **76,** 87-93.

Vainio, A. E. (1994) Effect of upstream sequences of the *ADH1* promoter on the expression of *Hormoconis resinae* glucoamylase P by *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **121,** 229-235.

van der Maarel, M. J., van der Veen, B., Uitdehaag, J. C., Leemhuis, H. and Dijkhuizen, L. (2002) Properties and applications of starch-converting enzymes of the α-amylase family. *J. Biotechnol.* **94,** 137-155.

Van der Vaart, J. M., te Biesebeke, R., Chapman, J. W., Toschka, H. Y., Klis, F. M. and Verrips, C. T. (1997) Comparison of cell wall proteins of *Saccharomyces cerevisiae* as anchors for cell surface expression of heterologous proteins. *Appl. Environ. Microbiol.* **63,** 615-620.

Wald, M. L. (2007) Is ethanol for the long haul? *Sci. Amer.* Jan, 42-48.

Washida, M., Takahashi, S., Ueda, M. and Tanaka, A. (2001) Spacer-mediated display of active lipase on the yeast cell surface. *Appl. Microbiol. Biotechnol.* **56,** 681-686.

Wong, D. W., Batt, S. B. and Robertson, G. H. (2001) Characterization of active barley α-amylase 1 expressed and secreted by *Saccharomyces cerevisiae*. *J. Protein Chem.* **20,** 619-623.

Wong, D. W., Batt, S. B., Tibbot, B. K. and Robertson, G. H. (2000) Isolation of a raw starch-binding fragment from barley α-amylase. *J. Prot. Chem.* **19,** 373-377.

Xiao, Z., Storms, R. and Tsang, A. (2006) A quantitative starch-iodine method for measuring α-amylase and glucoamylase activities. *Anal. Biochem.* **351,** 146-148.

Zhao, H., Shen, Z. M., Kahn, P. C. and Lipke, P. N. (2001) Interaction of α-agglutinin and a-agglutinin, *Saccharomyces cerevisiae* sexual cell adhesion molecules. *J. Bacteriol.* **183,** 2874-2880.

Zou, W., Ueda, M., Murai, T. and Tanaka, A. (2000) Establishment of a simple system to analyse the molecular interaction in the agglutination of *Saccharomyces cerevisiae*. *Yeast* **16,** 995-1000.

Zou, W., Ueda, M. and Tanaka, A. (2001) Genetically controlled self-aggregation of cell-surface-engineered yeast responding to glucose concentration. *Appl. Environ. Microbiol.* **67,** 2083-2087.