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PRODUCTION, CHARACTERIZATION AND CLONING OF GLUCOAMYLASE FROM *LACTOBACILLUS AMYLOVORUS* ATCC 33621

by

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Suggested short title :

GLUCOAMYLASE FROM *LACTOBACILLUS AMYLOVORUS*

DEDICATION

To my husband, Dariusz, my daughter, Tiffany

and my parents Gloria and Kenneth

ABSTRACT

Glucoamylase, a saccharifying enzyme, is applied in the brewing industry to hydrolyse the dextrins of malted barley into simple sugars which can then be fermented by brewer's yeast. In order to establish the potential of glucoamylase from *Lactobacillus amylovorus* for application in the brewing industry, the main objectives of this study were: 1) to determine the cultural conditions for growth and glucoamylase production, 2) to purify the enzyme to homogeneity using chromatography and electrophoretic techniques, 3) to study biochemical properties of the purified enzyme, and 4) to clone the gene coding for glucoamylase, and characterize the recombinant glucoamylase.

The actively amylolytic *Lactobacillus amylovorus* ATCC 33621 produced an intracellular glucoamylase activity. Conditions for growth and glucoamylase production were maximized by using dextrose free MRS medium supplemented with 1% dextrin, at pH 5.5 and 37°C. Enzyme production was maximal during the late logarithmic phase of growth from 16-18 h. Crude cell extract showed optimal activity at pH 6.0 and 55°C.

Native and SDS-PAGE of the purified enzyme showed a monomeric protein of 47 kD. Glucoamylase activity was confirmed by activity staining using a starch/ polyacrylamide gel where a zone of clearing was visible on a blue/black background stained with KI/I_2. Optimal pH, pI and temperature of purified glucoamylase were 4.5, 4.39 and 45°C, respectively. The enzyme was rapidly

inactivated by temperatures above 55°C and was inhibited by heavy metals, e.g. Pb^{2+} and Cu^{2+} at 1.0 mM. EDTA did not inhibit the enzyme activity at a final concentration of 10 mM. Enzyme inhibition by 1 mM of *p*-chloromercuribenzoic acid (pCMB) and iodoacetate suggested that a sulfhydryl group was present in the enzyme active site. Kinetic studies of glucoamylase confirmed that the enzyme reacted preferentially with polysaccharides. HPLC analyses of the end products of enzyme action showed that glucose was the major end product of enzyme action and this glucose was responsible for end product inhibition.

The gene coding for glucoamylase was cloned into *Escherichia coli* using the *STA2* glucoamylase gene of *Saccharomyces diastaticus* as a probe. Three glucoamylase producing transformants were identified as the insert sizes of about 5.2 Kb, 6.4 Kb and 5.9 Kb, respectively. When the characteristics of both recombinant and purified wild type glucoamylases were compared, both enzymes showed a similar pH range of 3.0-8.0, and temperature optimum of 45°C. The recombinant enzyme pH profiles were broader than that of the wild type and an optimum pH of 6.0 was obtained. This study has shown that glucoamylase from *Lb. amylovorus* is less heat stable than other bacterial glucoamylases and thus may be suitable for application in the brewing industry. Successful cloning of this gene coding for glucoamylase in brewer's yeast, *Saccharomyces cerevisiae*, would reap the advantageous properties of the enzyme while eliminating the costs of adding commercial enzymes.

RÉSUMÉ

PRODUCTION, CHARACTERIZATION ET CLONAGE DE GLUCOAMYLASE DE *LACTOBACILLUS AMYLOVORUS* ATCC 33621

La glucoamylase, un enzyme saccharolyrique, est utilisée dans les brasseries pour hydrolyser les dextrines du malt d'orge et ainsi produire les sucres simple que la levure fermente par la suite. Afin d'établir le potentiel de la glucoamylase provenant de *Lactobacillus amylovorus* dans une telle optique, la présente étude avait comme objectifs de: (1) déterminer les conditions de culture pour la croissance du microorganisme et la production de la glucoamylase, (2) purifier l'enzyme à l'aide des techniques de chromatographies et d'électrophorèses, (3) étudier les caractéristiques de l'enzyme ainsi purifié, (4) effectuer le clonage du gène encodant pour la glucoamylase, et caractériser la glucoamylase recombinante.

La souche amylolytique active, *Lactobacillus amylovorus* ATCC 33621 a démontré une activité glucoamylasique de type intracellulaire. L'utilisation d'un milieu MRS sans dextrose mais enrichi avec 1% de dextrine à pH 5,5 et 37°C a permis d'optimiser les conditions de culture et de production de l'enzyme. Ce dernier paramètre a atteint un maximum durant la phase logarithmique tardif, de 16-18 heures. Une activité optimale à pH 6,0 et 55°C a été notée pour un extrait cellulaire brut obtenu dans ces conditions.

L'étude à l'aide des techniques d'électrophorèse conventionelle et en conditions dénaturantes (avec SDS), a permis d'identifier une protéine

monomérique d'une masse moléculaire de 47 kD. Quant à la présence d'activité glucoamylasique, ceci fut confirmé par coloration active à l'aide d'une gel de polyacrylamide/amidon dans lequel une zone claire était visible sur fonds bleu/noir coloré avec KI/I_2. Le pH optimal, le point isoélectrique ainsi que la température optimale ont été estimés à 4,5, 4,39 et 45°C respectivement. L'enzyme était rapidement inactivé aux température excédant 55°C et l'addition de métaux lourds Pb^{2+} et Cu^{2+} à une concentration de 1,0 mM a également inhibé l'activité. EDTA n'a pu causer d'inhibition même à une concentration finale de 10 mM. Par contre, l'effet du *p*-chloromercuribenzoate (*p*CMB) et de l'iodoacétate à 1 mM suggère la présence d'une groupe sulfhydryle sur le site actif de l'enzyme. L'étude cinétique de la glucoamylase a révélé que cet enzyme réagit de façon préférentielle avec les glucides à longues chaînes. L'analyse des produits de la réaction par chromatographie HPLC a identifié le glucose comme principal produit; par ailleurs ce même glucose est responsable d'inhibition par le produit.

Le gène encodant pour la glucoamylase a été cloné dans *Escherichia coli* en utilisant le gène glucoamylasique *STA2* de *Saccharomyces diastaticus* comme sonde. Trois transformants exprimant une activité glucoamylasique ont été identifiés avec insertions de plasmides d'ADN d'environ 5,2 Kb, 6.4 Kb et 5,9 Kb. Quands les charactéristiques de glucoamylase des clones recombinants et celles d'enzyme purifié de *Lb. amylovorus* ont été comparées, les deux enzymes ont atteint un pH similair entre 3.0-8.0 et une temperature optimale de 45°C. L'enzyme de clones recombinants a eu un plus large profile que celui du natif et un pH

optimal de 6.0. La présente étude a démontré que la glucoamylase de *Lb. amylovorus* est moins thermostable que les autres glucoamylases des bactéries, et de ce fait pourrait s'appliquer dans les brasseries. La réussite du clonage de ce gène glucoamylasique dans *S. cerevisiae* permettrait de mettre à profit les propriétés avantageuses de cet enzyme tout en réduisant l'ajout d'enzymes commerciaux.

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PRAISE BE TO GOD. WITH GOD ALL THINGS ARE POSSIBLE!

FOREWORD

This thesis is submitted in the form of original papers suitable for journal publication. The first section is a general introduction and literature review presenting the theory and background information on this topic. The next four sections represent the body of the thesis, each being a complete manuscript. The last section is a summary of the major conclusions. This thesis format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions outlined in the Guidelines concerning Thesis Presentation, section 7 "manuscripts & Authorship" which are as follows:

"The candidate has the option, subject to the approval of the Department, including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication. In this case, the thesis must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional material (experimental and design data as well as descriptions of equipment) must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported. The abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstracts, introductions and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted."

While the inclusion of manuscripts co-authored by the candidate and others is not prohibited by McGill, the candidate is warned to make an explicit statement on who contributed to such work and to what extent, and Supervisors and others will have to bear witness to the accuracy of such claims before the oral committee. It should also be noted that the task of the External Examiner is made more difficult in such cases, and it is in the Candidate's interest to make authorship responsibility perfectly clear.

Although all the work reported here is the responsibility of the candidate, the project was supervised by Dr. Byong H. Lee, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

PREFACE

Claim of Original Research

1. This was the first study to identify glucoamylase from food grade bacteria, especially from lactic acid bacteria.
2. The conditions for maximal growth and glucoamylase production from *Lactobacillus amylovorus* ATCC 33621 were established. This actively amylolytic strain was identified as a source of an intracellular glucoamylase.
3. Glucoamylase from *Lactobacillus amylovorus* was purified to homogeneity using ion exchange chromatography and gel filtration columns of the Fast Protein Liquid Chromatography system (FPLC), followed by preparative gel electrophoresis. This allowed assessment of its amino acid composition, N-terminal amino acid sequence, apparent molecular weight and iso-electric point.
4. The enzyme was characterized sufficiently to allow assessment of its potential application in the brewing industry. The optimal pH and temperature of the crude glucoamylase were 6.0 and 55°C respectively. The enzyme was rapidly inactivated at temperatures above 55°C and thus, this is the first thermolabile bacterial glucoamylase to be identified.
5. The action of glucoamylase on polysaccharides of varying length was investigated by estimating kinetic constants. The enzyme preferentially digested large polysaccharides like starch and dextrin, thus demonstrating its suitability for application in the brewing industry.

6. This is the first study to clone the glucoamylase gene from a *Lactobacillus* strain in *Escherichia coli*. A non radioactive probe prepared from the *Saccharomyces diastaticus* glucoamylase STA2 gene was used.
7. The recombinant glucoamylase from positive clones was characterized and evidence has shown that the pH and temperature profiles of the recombinant and wild-type proteins are similar.

A. Part of this work has been published as follows:

James, J.A. and Lee, B.H. 1995. Cultural conditions for production of glucoamylase from *Lactobacillus amylovorus* ATCC 33621. J. Applied Bacteriol., 79, 499-505.

James, J.A. and Lee, B.H. 1996 .Characterization of glucoamylase from *Lactobacillus amylovorus*. Biotechnol. Lett. 18(12) 1401-1406.

James, J.A., Robert, N. and Lee, B.H. 1996. Cloning of glucoamylase from *Lactobacillus amylovorus*. Biotechnol. Lett. 18(12) 1407-1412.

James, J.A., Berger, J.L. and Lee, B.H. 1997. Purification of glucoamylase from *Lactobacillus amylovorus*. Current Microbiol. 34(3),.

James, J.A. and Lee, B.H. 1997. Glucoamylases: Microbial sources, industrial applications and cloning. Food Biochemistry (in press).

B. Part of this work has been presented at Scientific Conferences:

James, J.A. and Lee, B.H. 1994. Characterization of glucoamylase from *Lactobacillus amylovorus* ATCC 33621. Institute of Food Technologists (IFT) 55th. Annual Meeting and Food Expo, Atlanta, Georgia (June 25-29). **This work has been awarded first prize in the Graduate students' paper competition, Food Biotechnology Division of the Institute of Food**

Technologists.

James, J.A. and Lee, B.H. 1994. Glucoamylase from *Lactobacillus amylovorus* ATCC 33621. Canadian Institute of Food Technology (CIFST), Annual Conference, Vancouver (May 15-18).

James, J.A. and Lee, B.H. 1995. Properties of glucoamylase from *Lactobacillus amylovorus* ATCC 33621: Characterization and amino acid sequence. IFT 56th. Annual Meeting and Food Expo, Anaheim, California (June 2-7).

James, J.A. and Lee, B.H. 1995. Analysis of glucoamylase from *Lactobacillus amylovorus* ATCC 33621. Canadian Institute of Food Technology (CIFST), Annual Conference, Vancouver (July 7-11).

James, J.A., Berger, J.L. and Lee, B.H. 1996. Étude de la glucoamylase de *Lactobacillus amylovorus*. 64^{e} Congrès de l'Association Canadienne -Française pour l'avancement des Sciences (ACFAS), Montréal (May 13-17).

James, J.A., Berger, J.L. and Lee, B.H. 1996. Action of glucoamylase from *Lactobacillus amylovorus* and its cloning in *Escherichia coli*. IFT 57th. Annual Meeting and Food Expo, New Orleans, Louisianna (June 22-25).

Permission to use part of this material has been obtained from the co-authors of the manuscripts listed above.

GENERAL INTRODUCTION

Glucoamylase (EC 3.2.1.3) is an exo-acting carbohydrase which liberates ß-D-glucose from the non-reducing chain-ends of oligosaccharides by hydrolysing α-1,4 linkages consecutively. The α-1,6 glycosidic bonds are also hydrolysed, but at a much reduced rate. Glucoamylases have been applied in dextrose production, the baking industry, the animal feed industry, and in whole grain hydrolysis for alcohol production. The primary application of glucoamylases commercially is the production of glucose syrups from starch. Glucoamylases have also been applied in the production of low calorie beer where dextrins in malted barley are hydrolysed into simple sugars which can then be completely fermented by brewers yeast. Yeast can only ferment maltose, maltotriose, sucrose, glucose and fructose, and the complex dextrins which are still present in malted barley or brewer's wort would remain in beer, thus increasing overall calories.

The typical glucoamylase of choice in the food industry is the thermostable enzyme obtained from mold strains, since this permits rapid reactions at high temperatures, especially in the production of glucose syrups. In low calorie beer production, however, there is a need for a thermolabile glucoamylase which can be more readily inactivated by mild pasteurization (60-65°C, 6 min/ 71-74°C, 15 sec) used in the final stage of beer manufacture. The mold enzymes used commercially, tend to be thermostable, with residual activity lingering after production, resulting in undesirably sweet beer during storage.

Another microbial source of glucoamylase, or the cloning of the microbial gene coding for glucoamylase into Brewer's yeast (*Saccharomyces cerevisiae*), would be of great benefit, eliminating the addition of commercially prepared enzymes and thus reducing overall production costs. A great deal of research has been done since the early 1980's to find the perfect yeast clone which could hydrolyse dextrins as well as ferment sugars. Since worldwide sales of alcoholic beverages estimate over $67 billion per annum (Lee, 1991a; 1996), such research to provide cost saving measures, and improve fermentation technology, has been justified.

As an alternative source of glucoamylase, bacteria would provide the advantage of a shorter generation time (24 h versus three to five days) than molds. Glucoamylase activity has been demonstrated in a few bacteria like *Clostridium* sp. G0005 (Ohnishi *et al.* 1991) but these enzymes tend to be thermostable. There is the additional concern of the organisms not being generally regarded as safe (GRAS) in the food industry (Potter, 1986). One group of prokaryotes, lactobacilli, have found many applications in the food fermentation industry. Moreover, *Lactobacillus amylovorus*, first isolated from cattle waste-corn fermentations (Nakamura 1981) is known to secrete large quantities of α-amylase and other uncharacterized amylolytic enzymes (Burgess-Cassler and Imam, 1991; Fitzsimons and O'Connell, 1994). Its active amylolytic ability makes this strain a good candidate for the investigation of glucoamylase production and for ascertaining future applications in the brewing industry.

In this study, intracellular glucoamylase production in *Lb. amylovorus* was obtained, and conditions for maximizing production of glucoamylase were investigated. The enzyme was purified and physical and biochemical properties were examined, to identify the enzyme's potential in the food industry, particularly with respect to its thermostability. Kinetics and substrate specificity studies were done. This glucoamylase was inactivated at temperatures above 55°C, and it reacted preferentially with polysaccharides. Thus, a potential for application in the brewing industry was identified and initial investigation of cloning of the glucoamylase gene from *Lb. amylovorus* in *E. coli* was achieved.

Erratt and Nasim (1989) patented a method for the cloning of a thermolabile glucoamylase gene from *Saccharomyces cerevisiae* var. *diastaticus* into *S. cerevisiae*. This glucoamylase was found to be temperature labile between 50° and 60°C. The glucoamylase gene of the cell bound glucoamylase of *Clostridium* sp. G0005 was the first bacterial glucoamylase to be cloned (Ohnishi *et al.* 1992). To date, the cloning of a glucoamylase gene from a food grade bacteria has not been reported. We investigated the glucoamylase gene coding for glucoamylase in *Lb. amylovorus* to determine whether the properties of this enzyme could be transferred to *Escherichia coli*, and that may offer a potential for cloning in brewer's yeast.

The main objectives of this study were:

(1) To determine the location of glucoamylase activity in *Lactobacillus*

amylovorus and maximize the conditions of growth and glucoamylase production by investigating effects of temperature, pH and dextrin concentration.

(2) To purify glucoamylase from *Lactobacillus amylovorus* and estimate its molecular mass, isoelectric point, as well as amino acid composition and the N-terminal amino acid sequence of the glucoamylase active fraction.

(3) To characterize glucoamylase by investigating kinetics (K_m,V_{max}), substrate specificity, thermostability and the effect of inhibitors/activators.

(4) To clone the glucoamylase gene from *Lactobacillus amylovorus* in *Escherichia coli*, prepare a restriction enzyme map of the gene coding fragment, and to compare the characteristics of recombinant enzyme with those of the wild-type enzyme.

CHAPTER 1.0

LITERATURE REVIEW

GLUCOAMYLASES: MICROBIAL SOURCES, INDUSTRIAL APPLICATIONS AND CLONING

This chapter was summarized as the publication "Glucoamylases: Microbial sources, Industrial Applications and Molecular Biology - a Review" by Jennylynd A. James and Byong H. Lee, submitted to the Journal of Food Biochemistry. This paper was written by Jennylynd James and supervised by Dr. Byong Lee, who acted in an editorial capacity, evaluating the manuscript prior to submitting it to the journal. We referred to several references written by Byong H. Lee in this publication. The chapter serves as an introduction to the thesis, showing the background for the research that was done and the reasons why this project was significant.

1.1 ABSTRACT

Glucoamylase (EC 3.2.1.3) hydrolyses polysaccharides from the nonreducing chain ends by cleaving α-1,4 and α-1,6 glycosidic bonds consecutively. Glucoamylases are used mainly in the production of glucose syrup, high fructose corn syrup, and in whole grain and starch hydrolysis for alcohol production. This

paper reviews the status of glucoamylases with respect to microbial sources, biochemical and physical properties. Methods used to assay glucoamylase activity are also compared, with reference being made to the specificity of spectrophotometric methods in detecting end products of the enzyme action. Commercial glucoamylases and development of immobilization techniques are discussed. Also, structural analysis of glucoamylase and the main amino acids involved in catalysis and starch binding are emphasized. The cloning of the glucoamylase gene in *Saccharomyces cerevisiae* for the brewing of low calorie beer is presented. This review highlights the use of glucoamylase in the food industry.

1.2 DEFINITION

Glucoamylase is an exo-acting enzyme which breaks down starch into its component units, ß-D-glucose. Glucoamylase has been loosely called amyloglucosidase, glucamylase or l-amylase (Solomon, 1978). A more technical name is α-(1→4) glucan glucohydrolase where glucan is the name for a series of glucose units attached and glucohydrolase describes the breaking of the bond between two glucose units. EC 3.2.1.3 is the enzyme code assigned by the Enzyme Commission (IUBM, 1992). The number 3 denotes hydrolases which catalyze the hydrolytic cleavage of large molecules with the addition of water. The number 2 indicates glycosidases, those enzymes which catalyze the cleavage of a glycosidic

bond. Glycosidases are further subdivided into those hydrolysing O-glucosyl compounds, the group 3.2.1. There are several enzymes within this group, of which glucoamylase (EC 3.2.1.3) is number 3 (IUBM, 1992).

1.3 ACTION OF GLUCOAMYLASE

1.3.1 Starch Structure

In order to understand the action of glucoamylase, we must first review the structure of starch. Starch is made up of two main macromolecules, amylose and amylopectin in the proportion of one part amylose to three parts amylopectin (depending on the source). Both high molecular weight polysaccharides are organized in semicrystalline granules (diameter 2-100 μm), where both macro molecules are involved in crystallinity (Mercier and Colonna, 1988). Amylose is made up of glucose units linked by an α-1,4 -glycosidic bond. The carbon 1 of a glucose unit is linked to carbon 4 of the other glucose unit (Fig.1.1). The bond is rotated downwards, hence the name α-1,4 bond. The amylose polymer forms a chain and could be made up of almost 500 glucose units (Solomon, 1978; Mercier and Colonna, 1988).

In amylopectin, there is the same α-1,4 glycosidic linkage between two glucose units but in addition at branch points there is an α-1,6 linkage where C1 on

one chain is linked to C6 to form a branch point in the chain. There is thus a branched, bushlike structure which contains 3 to 5% branching linkages of α-1,6 glycosidic bonds. Both amylose and amylopectin are heterogeneous with respect to their molecular weight.

1.3.2 Carbohydrases

Carbohydrases are those enzymes which are able to break down starches and other oligosaccharides (Fig. 1.2). α-Amylase (E.C.3.2.1.1) hydrolyses α-1,4-glycosidic bonds randomly in amylose, amylopectin and glycogen in an endo-fashion. All α-amylases bypass α-1,6 glycosidic bonds, but do not cleave them. Hydrolysis of amylose by α-amylase causes its conversion into maltose and maltotriose, followed by a second stage in the reaction, the hydrolysis of maltotriose. Hydrolysis of amylopectin by α-amylase also yields glucose and maltose in addition to a series of branched α-limit dextrins each containing four or more glucose residues with the α-1,6 glucosidic linkages of the original structure. These enzymes require calcium as a cofactor and they can be subdivided into two groups: the liquefying α-amylases which preferentially degrade substrates of 15 or more residues; and the saccharifying α-amylases which degrade substrates of 4 or more residues. Commercial applications of α-amylases include the thinning of starch in the liquefaction process in sugar, alcohol, and brewing industries. Liquefaction is a

process of dispersion of insoluble starch granules in aqueous solution followed by hydrolysis using thermostable amylase (Mercier and Colonna, 1988, Vihinen and Mäntsälä, 1989).

ß-Amylase (E.C.3.2.1.2) occurs widely in higher plants. This enzyme acts in an exo-fashion from the non-reducing chain-ends of amylose, amylopectin or glycogen and hydrolyses alternate glycosidic linkages producing the ß-anomeric form of maltose. It is unable to bypass α-1,6 glycosidic linkages in amylopectin and glycogen, thus degradation is incomplete resulting in 50-60% conversion to maltose and formation of a ß-limit dextrin (i.e. the parent polymer with the outer chains trimmed down close to the outer most branch points) (Maeda and Tsao, 1979; Vihinen and Mäntsälä, 1989).

Glucoamylase is an exo-acting enzyme that yields ß-D-glucose from the non-reducing chain ends of amylose, amylopectin and glycogen by hydrolysing α-1,4 linkages in a consecutive manner (Fogarty, 1983). It also hydrolyses α-1,6 and the rare α-1,3 linkages although at a much slower rate. So this enzyme is able to completely hydrolyse starch if incubated for extended periods of time. It is called the saccharifying enzyme. Glucoamylases have the capacity to degrade large oligosaccharides up to about 90% α-1,6 linkages. However, the size of the substrate and the position of the α-1,6 linkages play a significant role in the susceptibility to hydrolysis. Reversion reactions involving resynthesis of saccharides from D-glucose may occur with high glucoamylase concentration for prolonged incubation time and

high concentrations of substrate (35-40%). The main reversion products are maltose and isomaltose. The pH and temperature optima of glucoamylases are generally in the range 4.5 - 5.0 and 46 - 60°C, respectively, with a few exceptions (Fogarty, 1983; Vihinen and Mäntsälä, 1989).

In addition, transglucosidase (EC 2.4.1.24) occurs as an impurity in crude preparations of glucoamylase. This enzyme catalyses the synthesis of nonfermentable glucose saccharides by transferring a glucosyl residue from an α-1,4 position to an α-1,6 position. Panose and maltose build up, thus affecting the final yield of glucose. A number of methods have been patented for the removal of transglucosidase from commercial glucoamylase preparations, the most successful being selection of mutants with low transglucosidase activity (Fogarty, 1983).

1.3.2.1 Hypotheses of glucoamylase action

It has been suggested that glucoamylase acts by a multichain mechanism in which the enzyme acts randomly on all the substrate molecules (Solomon, 1978). One mechanism which has been suggested for the cleavage of the glycosidic bond is illustrated in Fig. 1.3. The D-glycosidic bond oxygen is protonated by hydrogen ions from an amino group or imidazole group of the enzyme active site. The electron deficient center at C-1 of the bond attracts electrons from a donor group such as a hydroxyl group, either from water or a serine group in the active site. The resulting

structure is cleaved at the C-1 carbon side of the bond forming a carbonium ion intermediate and a neutral D-glucosyl fragment. The final step involves the addition of a hydroxyl ion (or a water molecule) to the carbonium ion intermediate. The OH group is added in the ß- configuration hence the name ß-D-glucose as the product of hydrolysis (Solomon, 1978). Further details on amino acid structure at the enzyme active site are reviewed in detail in a later section.

1.3.3 Glucoamylase assays and their limitations

Glucoamylase has been assayed by reaction with large oligosaccharides like starch, in solution, then measuring the amount of glucose released. One typical example is the assay carried out by Ueda *et al.* (1981), where the following enzyme-substrate mixture was prepared: 5 ml 1% soluble starch; 1 ml 0.1 M sodium acetate buffer, pH 4.5; 1 ml water, and 1 ml enzyme solution. After incubation at 40°C for 10 min, the amounts of reducing substances in a 1 ml mixture were determined by the Nelson-Somogyi method (Nelson, 1944). One unit of glucoamylase activity was defined as the amount of enzyme that formed 1 mg glucose in 1 ml reaction mixture under the assay conditions. Similar assays for glucoamylase have been used by other researchers (Mahajan *et al.*, 1983; Ghosh *et al.*, 1990; Kuchin *et al.*, 1993).

Although starch is frequently used as the substrate for glucoamylase assays, this substrate is also used to assay for α-amylase action. There is no guarantee that the glucose produced as a result of enzyme action is the product of only glucoamylase action. Other enzymes like α-amylase and ß-amylase may also assist in hydrolysing the starch and thus may have a synergistic effect on glucoamylase action (Saha and Zeikus, 1989). In addition, the assay for reducing sugars, the Nelson-Somogyi method, accounts for all the reducing sugars present and is not specific for glucose. One improvement on this method would be to use an enzymatic method for glucose detection. Test kits have been developed by Boehringer Mannheim, using hexokinase-glucose-6-phosphate dehydrogenase (HK/GDH) and glucose-6-phosphate dehydrogenase coupled assay with $NADP^+$ to measure hexoses (Bergmeyer *et al.* 1974). Test kits employing the enzyme glucose oxidase, for example the Glucose Trinder test kit produced by Sigma Chemical Co., is specific for glucose, and may thus improve the glucoamylase assay (Trinder, 1969). Using a complex substrate, then assaying for the product of enzyme action, is an indirect method of measuring glucoamylase activity.

Electrophoretic transfer has been used as a technique for the detection of amylolytic enzymes in polysaccharide gels (Kakefuda and Duke, 1984; Krause *et al.* 1991). Various limit dextrins in the starch gel were identified by their characteristic color development in KI/I solution and this same gel may be used to identify α-amylase and ß-amylase. A high specific activity is required for detection of

glucoamylase in starch gels (Krause *et al.* 1991), and the protein band appears as a clear zone against the blue-black background stained with iodine. Pazur *et al.* (1971) reported a continuous glucoamylase assay which used the glucose oxidase/peroxidase assay with amylose as substrate and O-dianisidine as chromogen. Also an assay using maltotetraose as substrate and 3-dimethylaminobenzoic acid and 3-methyl-2-(3H)-benzothiazolinone hydrazone as chromogens, has been reported (Sabin and Wasserman, 1987). Kleinman *et al.* (1988) used a semi quantitative activity determination for glucoamylase involving the chromogenic substrate *p*-nitrophenyl α-D-glucopyranoside. Substrate, *p*-nitrophenyl α-D-glucopyranoside (10 µl of 1 mg/ml concentration) and 10 µl of enzyme solution were incubated overnight at 37°C. The yellow color (*p*-nitrophenol) caused by glucoamylase activity could be measured, after dilution, in a spectrophotometer at 400 nm.

McCleary *et al.* (1991) developed a test kit for the Megazyme Ltd. of Australia, based on the same principle using a chromogenic substrate *p*-nitrophenyl-ß-maltoside. The enzyme solution (0.2 ml) is incubated with 0.2 ml of reaction solution at 40°C for 10 min. The reaction is terminated by the addition of 3 ml of 2% Trizma base. The yellow color of *p*-nitrophenol formed as a result of glucoamylase activity is measured spectrophotometrically at 410 nm. ß-Glucosidase is present in saturating levels in the substrate preparation. When the terminal α-linked D-glucosyl residue is removed by glucoamylase, the ß-glucosidase gives immediate removal of

the ß-linked D-glucosyl residue, with the release of free *p*-nitrophenol. Pazur *et al.* (1971) developed a rapid, micro procedure of assaying for glucoamylase. The procedure is a coupled enzyme assay in which D-glucose oxidase and peroxidase are used to measure the D-glucose liberated from the reaction of glucoamylase with amylose. A chromogen (o-dianisidine) is used in the reaction mixture, and the u.v. absorption of the reduced chromogen is monitored continuously in a spectrophotometer at 400 nm. A change in absorbance of 1.0 per min was used to define enzyme units.

Glucoamylase hydrolysis of starch can be affected by other amylolytic enzymes such as α-amylase, ß-amylase, pullulanase, isoamylase or α-glucosidase, making estimation of activity difficult. Improvements have been achieved by detecting α-amylase contamination by using an oxidized starch (Saha and Zeikus, 1989). Pullulanase or isoamylase contamination can be readily avoided by using amylose as the substrate, and α-glucosidase contamination by measuring the difference in the enzyme activity using starch as the substrate, with or without maltose. A higher glucose yield in the latter case would reveal the presence of α-glucosidase (Saha and Zeikus, 1989).

1.4 SOURCES AND FORMS OF GLUCOAMYLASE

Glucoamylases can be derived from a wide variety of plants, animals and microorganisms, though most glucoamylases occur in fungi. The enzymes used commercially originate from strains of either *Aspergillus niger* or *Rhizopus sp.* where they are used for the conversion of malto-oligosaccharides into glucose (Fogarty, 1983; Pandey, 1995). These enzymes are generally regarded as safe (GRAS) by the Food and Drug Administration (FDA). The properties of glucoamylase have been reviewed comprehensively elsewhere (Vihinen and Mäntsälä, 1989). Since the discovery of two forms of glucoamylase from black koji mold in the 1950's, many reports have appeared on the multiplicity of glucoamylases. The various forms of glucoamylases are thought to be the result of several mechanisms: mRNA modifications, limited proteolysis, variation in carbohydrate content or the presence of several structural genes (Pretorius *et al.* 1991).

1.4.1 Mold Glucoamylases

There are between one and five forms of all fungal glucoamylases which tend to be glycoproteins. The filamentous fungus *Aspergillus* is an organism of considerable importance for a variety of biotechnological industries. In addition to their application in starch processing, several species have been used in the

production of secondary metabolites and several hydrolytic enzymes, glucoamylase being the most important. *Aspergillus niger* glucoamylase has been used widely in the commercial production of this extracellular enzyme. Two forms of glucoamylases were isolated from *A. niger*, glucoamylase I (99 kD) and glucoamylase II (112 kD) (Pazur *et al.* 1971). Both forms contained covalently linked carbohydrate (D-mannose, D-glucose and D-galactose residues). The carbohydrate-protein linkage in these glycoenzymes is mainly glycosidic to the hydroxyl group of L-serine and L-threonine residues but glucosylamine linkages to L-asparagine and L-glutamine may also be present. The two forms of the enzyme have been termed isoglycoenzymes. They had identical amino acid content but the number of amide groups or glycosylaminically linked units probably account for the electrophoretic differences (Pazur *et al.* 1971).

The two forms of glucoamylase (AG-I and AG-II) isolated from *A. niger* van Tieghem, however, differed in their carbohydrate content, as well as the pH and temperature stabilities and optima for activity (Ramasesh *et al.* 1982). Their activation energies and K_m values also varied and AG-II had higher carbohydrate content, with an increased acid tolerance. Other workers also purified and characterized glucoamylases from *Aspergillus niger* strains (Williamson *et al.* 1992a; Pandey and Radhakrishnan, 1993; Stoffer *et al.* 1993).

Glucoamylases from *Aspergillus terreus* strains were also examined to verify the properties which make it suitable for production of D-glucose and corn syrups

(Ghosh *et al.* 1990; Ali and Hossain, 1991; Ghosh *et al.* 1991). This organism released glucoamylase extracellularly and a catabolite derepressed mutant of *A. terreus* 4 was found to produce 1.8 times more glucoamylase than the parent strain, reacting with raw starch. Optimal glucoamylase activities at various temperatures and solution conditions are summarized in Table 1.1. The nutritional requirements and fermentation parameters for production of glucoamylase from *A. candidus* Link var. *aureus* have been reported (Kolhekar *et al.* 1985; Narayanan and Ambedkar, 1993). The partially purified enzyme gave 84% conversion efficiency of starch to glucose in 10% starch solution without the addition of a liquefying enzyme. Glucoamylase from this strain also showed an industrial potential because of the high specific activity and yields obtained after protein purification (Mahajan *et al.* 1983; Kolhekar *et al.* 1985).

An economical and efficient alternative to commercially available glucoamylase preparations was identified in glucoamylase from a mutant of *A. awamori*, which does not produce transglucosidase (Shah *et al.* 1989). The ingredients for production and fermentation parameters were optimized and the enzyme was concentrated 14-fold by ultrafiltration to give efficient saccharification of starch. Three forms of *A. awamori* var. *kawachi* glucoamylase were produced selectively in different media in the presence of zinc which seemed to stimulate proteases. The enzymes had the following molecular weights: GAI, 90,000; GAII, 83,000 and GAIII, 57,000 (Pandey, 1995). Properties of the degraded form were not

similar, but this is a good example of proteolysis being responsible for multiple forms of glucoamylase.

Rhizopus oryzae was reported as being capable of simultaneously saccharifying and fermenting corn starch and other cereals to L-lactic acid (Yu and Hang, 1991; Suntornsuk and Hang, 1994). Also, *Rhizopus* sp. generally do not produce transglucosidase and thus have been widely used in the Amylo-process for conversion of starch to glucose with up to 100% efficiency (Yu and Hang, 1991). The main advantage of glucoamylase derived from *Rhizopus* is that it exhibits a strong action on raw starch and its chemical properties, including optimum pH are well suited to the saccharification of cereal starch (Ashikari *et al.* 1985). Three forms of glucoamylase were isolated from *Rhizopus* sp., Gluc 1, Gluc 2 and Gluc 3 (Takahashi *et al.* 1985). The pH optima for raw starch digestion were different and all the enzymes digested raw starch. Glucoamylases from other mold strains have been investigated for their thermotolerant properties, for example enzyme from the strains *Humicola lanuginosa* (Taylor *et al.* 1978) and *Thermomyces lanuginosa* (Haasum *et al.* 1991). Thermostable enzymes are required for the production of glucose syrups. One phytopathogenic fungus which produces glucoamylase is *Colletotrichum gloeosporoides* (Krause *et al.* 1991).

1.4.2 Yeast Glucoamylases

The production of glucoamylase by yeast has been well reviewed (Erratt and Nasim, 1987; Saha and Zeikus, 1989, Pretorius *et al.* 1991). Glucoamylase has been identified in *Saccharomyces cerevisiae* (Pugh *et al.* 1989), *Saccharomyces cerevisiae* var. *diastaticus* (Kleinman *et al.* 1988; Pretorius *et al.* 1991), *Saccharomycopsis fibuligera* (Itoh *et al.* 1989), *Schwanniomyces castellii* (Sills *et al.* 1984), *Schwanniomyces occidentalis* (Gellissen *et al.* 1991), *Pichia burlonii* and *Talaromyces* sp. The expression of glucoamylase activity in *S. cerevisiae* is confined only to the sporulation phase of the life cycle, and is not regulated directly by composition of the growth medium (Pugh *et al.* 1989). Thus *S. cerevisiae* does not use starch and other complex oligosaccharides during growth and sugars have to be supplied in a simple form during fermentation for alcohol production. The sporulation glucoamylase, encoded by the SGA gene, is produced intracellularly to breakdown glycogen stores in the cell at the time of spore formation.

S. diastaticus is clearly allied to *S. cerevisiae* except that the former secretes extracellular glucoamylase. *S diastaticus* carrying any one of the unlinked *STA* genes (*STA* 1, 2, 3) produces extracellular glucoamylase isoenzymes I, II, III respectively (Lee, 1991b). Glucoamylase from a diastatic strain of *S. cerevisiae* was purified by chromatographic techniques and then characterized (Kleinman *et al.* 1988). The glucoamylase was heavily glycosylated and was found to be rich in

Threonine, Serine, Aspartic acid, and Asparagine residues. The kinetic behavior of this glucoamylase was similar to that of fungal glucoamylases: increasing hydrolytic rate with increasing maltodextrin chain length. The K_m values are usually high and V_{max} values low for substrates with α-1,6 linkages, such as isomaltose and pullulan.

1.4.3 Bacterial Glucoamylases

Some bacterial glucoamylases have been identified including aerobic strains such as *Bacillus stearothermophilus* (Srivastava, 1984), *Flavobacterium* sp. (Bender, 1981), *Halobacterium sodomense* (Oren, 1983; Chaga *et al.* 1993); *Arthrobacter globiformis* I42 (Okada and Unno, 1989). Anaerobic strains include *Clostridium thermohydrosulfuricum* (Hyun and Zeikus, 1985), *Clostridium* sp. G0005 (Ohinishi *et al.* 1991), *Clostridium acetobutylicum* (Chojecki and Blaschek, 1986; Soni *et al.* 1992), *Clostridium thermosaccharolyticum* (Specka *et al.* 1991), and the microaerophile, *Lactobacillus amylovorus*, (James and Lee, 1995). Most of the glucoamylases produced by these bacteria except *Lactobacillus amylovorus*, are thermostable and are thus potential enzymes for application in the saccharification of starch for glucose syrup production, where temperatures greater than 60°C are employed.

A unique glucoamylase was isolated from a *Flavobacterium* sp. by Bender (1981). This enzyme described as "cyclodextrin-degrading glucoamylase" was found

to be inducible and cell bound, degrading Schardinger dextrins to D-glucose (Fogarty, 1983). The enzyme differs from other glucoamylases in that it is stabilized in the presence of calcium and inactivated by EDTA. The pH optimum for activity was in the range of 5.5-6.5 with a temperature optimum of 55°C. Relatively high salt concentrations are required for glucoamylase activity in *Halobacterium* sp. and this thermostable enzyme had a higher affinity for soluble starch than glucoamylases of several mold strains (Chaga *et al.* 1993). Glucoamylase from the anaerobe *Clostridium* sp. G0005 was found to be cell bound (CGA) and the kinetic properties of the enzyme were typical of other glucoamylases (Ohnishi *et al.* 1992). Comparison of the amino acid sequence of the CGA precursor with known eukaryotic enzymes showed several regions of similarity, but there was little overall similarity of the primary enzyme structure.

Clostridium thermohydrosulfuricum has been identified as a source of a thermostable glucoamylase which has potential in the starch-processing industry (Hyun and Zeikus, 1985). Both the debranching enzyme, pullulanase and glucoamylase activities of this strain were stable and optimally active at 85 and 75°C, respectively. These enzymes allowed the hydrolysis of starch without significant α-amylase activity. Glucoamylase from anaerobic bacterium, *Clostridium thermosaccharolyticum* (Specka *et al.* 1991) was also thermostable, with optimal activity at 70°C, and thus may be useful in the syrup industry. The use of enzymes from *Clostridium* sp. in the food industry, however, may require special regulations,

since anaerobic *Clostridium* sp. are known to produce toxins which cause botulism (Potter, 1986).

1.4.4 Properties of glucoamylase

Fungal glucoamylases are the most abundant and they have been well characterized and studied. The molecular mass of fungal glucoamylases range from 26.85 Kd to 112 Kd and the optimal pH of most glucoamylases fall within the range of 4.5 to 5.5 (Fogarty, 1983; Vihinen and Mäntsälä, 1989). Glucoamylases are usually stable on the acid side of pH 7.0. However, one exception is glucoamylase from *Humicola lanuginosa* which is stable up to pH 11.0 (Taylor *et al.* 1978). The isoelectric points of glucoamylases fall in the wide range of pH 3.7-7.4 and this property is species specific. Optimal temperatures of glucoamylases range from 40-60°C, but certain thermophilic strains like *H. lanuginosa* (Taylor *et al.* 1978) and *Aspergillus niger* IMDCC No.1203 (Fogarty and Benson, 1983) have temperature optima of 70°C. Enzymes of *Thermomyces lanuginosa* (Erratt, J.A. and Stewart, 1981) and *Clostridium thermohydrosulfuricum* (Hyun and Zeikus, 1985) can also be considered thermostable.

Mold glucoamylases tend to be glycoproteins which have between 5 and 20% carbohydrate in the forms of glucose, glucosamine, mannose and galactose. These enzymes have tracts rich in Threonine and Serine which are ideal for

glycosylation where carbohydrates are bond by an O-glycosidic bond. For example, glucoamylase of *A. niger* was found to have 11 disaccharides with the structure 2-O-D-mannopyranosyl-D-mannose, 8 trisaccharides and 5 tetrasaccharides attached by (1-3) and (1-6) glycosidic bonds (Fogarty, 1983; Saha and Zeikus, 1989). Such arrangements of carbohydrate residues and proteins may account for some of the properties of glucoamylases, like stability at high and low temperatures, and the resistance to proteolysis. Removal of carbohydrate moiety reduces the stability and activity of the enzyme (Saha and Zeikus, 1989).

Glucoamylases are able to hydrolyse 88.5 - 100% of soluble starch but not all are able to digest raw starch, an advantage which would be beneficial in the starch processing industry. Glucoamylase from some *A. awamori* and *A. oryzae* strains are known to hydrolyse raw starch and the K_m values for raw starch differ for different forms of glucoamylase (Table 1) (Vihinen and Mäntsälä, 1989). The amino acid composition of mold glucoamylases show high contents of threonine and serine (Fogarty, 1983), however, the composition of bacterial glucoamylases varies from species to species.

During raw starch hydrolysis, synergistic behavior has been observed between some amylolytic enzymes. A synergistic action of glucoamylase and α-amylase was identified in enzymes from *Aspergillus* sp. K-27 and enzymes from other mold strains (Abe *et al.* 1988; Saha and Zeikus, 1989). Glucoamylase from this strain was thermostable and its action on the degradation of starch granules

was enhanced in the presence of α-amylase. Using 20 U of glucoamylase activity, there was 40% starch hydrolysis in 9 h. When 0.2 U of α-amylase activity was added to 20 U of glucoamylase, the reaction increased 1.8 fold and, with the addition of 5 U α-amylase, the reaction rate increased 2.9 fold in 3 h (Abe *et al.* 1988). Addition of isoamylase accelerated waxy corn starch hydrolysis by glucoamylase of black koji mold and pullulanase from *Aerobacter* stimulated raw starch digestion by *Rhizopus* glucoamylase 1 (Saha and Zeikus, 1989). This synergistic action of α-amylase and glucoamylase on the hydrolysis of soluble starch has been modeled by kinetic equations. Early in the reaction, α-amylase contribute to the formation of new non-reducing chain ends, but, after the molecular weight of the substrate decreases to about 5,000, only the rate equation for glucoamylase is used (Fujii and Kawamura, 1985).

In the review of cyclodextrin degrading enzymes (Saha and Zeikus, 1992), some glucoamylases with the ability to hydrolyse cyclodextrin have been identified. In particular, glucoamylase produced by *Flavobacterium* sp. (Bender, 1981), a cell bound enzyme degrades amylose, amylopectin and glycogen (41%, 15% and 0.7%), respectively compared with cyclodextrin (100% hydrolysis). Cyclodextrin-hydrolysing enzymes may be applicable to industrial production of linear G6, G7 and G8 sugars from cyclodextrins.

Acarbose (BAY 5421), an oligosaccharide analog synthesized by BAYER of Germany, is one of the most potent inhibitors of glucoamylase. This compound was

developed to inhibit digestive enzymes like sucrase and maltase and consists of an aminocyclitol unit and a 6-deoxyhexose unit (Fig. 1.4). These analogs are able to fit into the active site, terminating further action on oligosaccharides. The dissociation coefficient, K_d of acarbose is 10^{-12} M (Svensson and Sierks, 1992). It contains a valienamine residue at its "non-reducing" end and a 4-amino-4,6-dideoxy-α-D-glucopyranose in place of the second glucose residue. Another potent inhibitor of glucoamylase is 1-deoxynojirimycin. The inhibition coefficient of deoxynojirimycin is K_i=96 μmol l^{-1} and the presence of nitrogen in the carbon ring is sufficient to inhibit glucoamylase activity (Harris *et al.* 1993).

No metal ion has been identified as a requirement for glucoamylase activity as compared to α-amylase which requires Ca^{2+} ions. The role of Ca^{2+} varies, in that this ion stimulates glucoamylase from *Schizophyllum commune*, while in large concentrations it inhibits glucoamylase from *Rhizopus delemar* (Vihinen and Mäntsälä, 1989). Heavy metals have been found to inhibit glucoamylases. For example Hg^{2+}, Pb^{2+} and Cd^{2+} inhibited glucoamylase from *R. oryzae*, Zn^{2+}, Cu^{2+} and Pb^{2+} inhibited enzyme from *C. thermosaccharolyticum*. The metal chelator EDTA does not seem to have any inhibitory effect. Starch stabilizes enzymes from *A. niger*, *C. thermohydrosulfuricum* and *H. lanuginosa* (Vihinen and Mäntsälä, 1989). Glucoamylase has been found to react selectively on large oligosaccharides. The rate of reaction increases with an increase in oligosaccharide chain length (Erratt and Nasim, 1987; Ali and Hossain, 1991; Specka *et al.* 1991; Pandey, 1995), thus

the K_m values for starch tended to be lower than the values for smaller oligosaccharides.

The total conversion of starch into D-glucose is only complete if a large excess of glucoamylase is allowed to act over long periods of time. As the hydrolysis of starch solution progresses, the initial rate of hydrolysis decreases continuously because of low affinity for α-1,6 linkages as well as product inhibition by glucose. Reversion reactions, catalysed by transglucosidase (EC 2.4.1.24), may also occur involving the resynthesis of saccharides like maltose and isomaltose. When glucoamylase is used in industrial processes, the substrate concentration is kept low (about 10%) and the reaction is terminated before equilibrium is reached, in order to obtain a high final glucose concentration (95%) (Fogarty, 1983).

1.4.5 Methods of purification of glucoamylase

The purification of glucoamylase is a necessary prerequisite for a full understanding of the nature and mechanism of its action. Glucoamylase was first obtained in a pure form by Pazur and Ando (1959). They used chromatography on DEAE-cellulose to purify extracts of mycelium of *A. niger*, and two forms (isoenzymes) of glucoamylase were obtained. These forms were separated by electrophoresis. Glucoamylase has been purified by procedures involving column

fractionation, including ion exchange, hydrophobic interaction and gel chromatography. Glucoamylase was also be purified from contaminating enzymes by adsorption of the impurities on naturally occurring acid clays, such as bentonite (Saha and Zeikus, 1989).

Ono *et al.* (1988) purified glucoamylase of *A. oryzae* by extraction with 1% NaCl solution, precipitation with ethanol and acarbose affinity chromatography. The purified protein was a homogeneous glycoprotein containing about 4.8% glucosamine and 7.8% neutral saccharides. Five forms of glucoamylase from *R. niveus* were separated by preparative isoelectric focusing in order to study the raw starch adsorption, elution and digestion behavior (Saha and Ueda, 1983). Takeda *et al.* (1985) studied the purification and substrate specificity of glucoamylase of *Paecilomyces varioti* AIIU 9417. The enzyme was purified by precipitation with ethanol, chromatography on DEAE-Sepharose CL-6B, gel filtration and preparative disc electrophoresis.

A simple and rapid method for the purification of glucoamylase from *Aspergillus terreus* based on the affinity for the carbohydrate moiety of the enzyme has been reported by Ali and Hossain (1991). Glucoamylase G2 from *Aspergillus niger* was purified by an affinity gel with a covalently-linked acarbose ligand (Clarke and Svensson, 1984b). The efficient binding of glucoamylase I from *A. awamori* var. *kawachi* to cyclodextrins and raw starch has been exploited in the production of an affinity column of α-Cyclodextrin-Sepharose CL-6B for purification (Vretblad, 1974;

Hermanson *et al.* 1992; Goto *et al.* 1994b). Different methods were used for purification of glucoamylase of different species specific, since one method used to purify glucoamylase from one organism may not necessarily work for other glucoamylases.

1.5 INDUSTRIAL APPLICATIONS OF GLUCOAMYLASES

Glucoamylase is second to protease in worldwide distribution and sales among industrial enzymes. Glucoamylases find many applications in industry. This enzyme is used in dextrose production, in the baking industry, in the brewing of low calorie beer and in whole grain hydrolysis for the alcohol industry.

1.5.1 Starch hydrolysis for glucose production

The most important application of glucoamylase is the production of high-glucose syrups. Other dextrose products formed as a result of glucoamylase action are high fructose corn syrup and high conversion syrups. The conversion of starch to sugars is one of the most important biotechnological processes with an annual output of over 8 million tons (Lee, 1991a). Initially D-glucose was prepared from starch by acid hydrolysis as discovered by Kirchoff in 1811 and de Saussure in

1815. However it was found that yields were low and reversion reactions considerable. Today, starch is hydrolysed by a two stage process: liquefaction then saccharification using a mixture of amylolytic enzymes :- thermostable α-amylase followed by glucoamylase (Finn, 1987; Lee, 1991a). The first step called liquefaction, involves treatment with α-amylase at 85-100°C. The dextrin is soluble and so can be passed through a bed of immobilized glucoamylase which accomplishes further hydrolysis to glucose units. High glucose syrups contain 96-98% D-glucose. They may be used directly for example in fermentation but transport and storage require heated conditions to prevent crystallization and solidification. High glucose syrups are used either for production of crystalline D-glucose or as a starting material for the production of high-fructose syrups. In both applications the highest possible concentrations of glucose are required to improve yields in the case of crystalline D-glucose and to reduce the amount of glucose isomerase required to yield a certain fructose level (Fogarty, 1983).

High conversion syrups have a dextrose equivalent (DE) 60-70, comprising 35-43% glucose, 30-47% maltose and 8-15% maltotriose. These syrups have a maximum permissible glucose concentration of about 43%. Any higher than this and glucose may crystallize out. These syrups are used in the brewing and baking industries as adjuncts of fermentable sugars. They are used as sweeteners in the soft drinks and canning industries, and the confectionery industry (Fogarty, 1983).

High value is placed on thermal stability and thermoactivity of glucoamylase to be used in the processing of starch to glucose syrups.

1.5.2 High Fructose Corn Syrup (HFCS) production

High fructose corn syrup (HFCS) has replaced cane or beet sugar in many important applications because fructose is sweeter than sucrose (Finn, 1987). Sales of HFCS have exceeded 4 million tons per annum, accounting for over 30% of the US sweetener market (Lee, 1991a). The following procedure is used in the production of high glucose syrup, then high fructose syrup from corn starch. An aqueous slurry of starch (35-45%DS) is liquefied and partially hydrolysed using a bacterial thermostable α-amylase. After liquefaction, the slurry is adjusted to pH 4.5 and the temperature is lowered to about 60°C. Glucoamylase, the saccharifying enzyme, is then added to produce glucose. Several stages of purification follow: filtration, carbon refining, ion exchange chromatography; followed by light evaporation to reach the required glucose concentration (96-98%), (Fig.1.5). This high glucose syrup is then fed to the isomerization tank where immobilized glucose isomerase is used at 60-65°C to produce and equilibrium mixture of glucose and fructose (Finn, 1987). Further purification steps follow, to obtain a highly refined high fructose corn syrup. This is one area where waste corn can be used to produce a high value commodity. The use of immobilized glucose isomerase for continuous

HFCS production is one of the earliest examples of a large-scale industrial use of an immobilized enzyme (Lee, 1991a).

The tubers cassava and potato may also be processed to make glucose and HFCS but few references are available for these reactions. Tapioca starch, from cassava, could be treated similarly with α-amylase for liquefaction and glucoamylase for saccharification, to form glucose (Lee, 1991a). Whole potato has also been successfully converted to glucose, maltose and soluble oligosaccharides by multiple enzyme treatment. Since 55% HFCS cost 15-20% less than an equal amount of sucrose syrup, the soft drink beverage industry has switched to its use. 90% HFCS is mainly used in low calorie foods because of its high sweetness.

1.5.3 Applications in the baking industry

Glucoamylases are also used in commercial baking. Enzymes have been used in the baking industry to improve flour quality, retard dough staling and improve dough, giving more efficient machinability. They have also been used to enhance bread crust color, bleach flour, improve the quality of high-fiber baked products and reduce the phytate content in whole-grain formulations (James and Simpson, 1996). Fungal and bacterial enzymes which are commercially available for use in bakery processing include α- and ß-amylase, proteases, glucoamylases, pentosanases and other enzymes.

Flour, water, yeast and other ingredients are mixed together to a spongy consistency. It is at this stage that α-amylase and glucoamylase may be added. The mixture is left to ferment at 29°C for 4 h. More flour is added and mixed to form the dough which is divided, proofed at 25°C for 12 min, molded and put in pans for a final proofing. Bread is baked at 215°C for 20 min.

Glucoamylases are added along with amylases directly to flour to assist in the conversion of starch to maltose and fermentable sugars. Fermentable sugars are used by yeast during proofing and baking to generate carbon dioxide which causes the dough to rise. Flour has only about 1-2% sugar and the native α- and ß-amylases may be insufficient for the breakdown of starch, hence the need for supplementation, especially with α-amylase. Staling occurs when starch molecules retrograde i.e. revert from a soluble to an insoluble crystalline form. This change redistributes water, leaving baked products hard and brittle. The addition of α-amylase and glucoamylase, it is suggested, may cleave a few bonds in the soluble region of starch, leaving the insoluble regions separated by those that can "flex". Another theory is that the amylopectin chain length is shortened from 19-21 units to 12-15 units, reducing the tendency to retrograde, also limiting crystal size. Thus shelf-life of bread is extended by 2 to 3 days.

1.5.4 Starch and whole grain hydrolysis for alcohol production

Low quality grains and starch containing waste materials which are surplus agricultural commodities, could be used to produce value added chemicals like fuel ethanol, methane and lactic acid (Lee, 1991a; Linko and Wu, 1993). Countries like Brazil, Canada, South Africa and the United States have a large agricultural surplus annually and studies are being conducted on the production of ethanol from sucrose, starch and plant biomass. About 66% of the starch in the United States is derived from maize of which 80% is converted to dextrose or HFCS. Corn dextrose is also further converted by fermentation with yeast, into fuel-grade ethanol which is used as an octane booster in lead-free gasoline (Lee, 1996). Processes are in operation to convert starch waste enzymatically into mono- and di-saccharides which are then fermented by yeast or *Zymomonas* bacteria into alcohol.

The first step in the process is the liquefaction of starch with α-amylase, followed by saccharification with glucoamylase to form a high yield of glucose. Debranching enzymes like isoamylase and pullulanase could be added to improve glucose yields (Linko and Wu, 1993). In the typical fermentation process, glucose syrup is mixed with corn steep liquor, a by-product of wet milling. The fermentation of glucose to alcohol is complete but the fermentation broth has to be concentrated and purified from 8-9% to 99.5% ethanol in order to blend with gasoline (Lee, 1996). At some companies, yeast cells are centrifuged from the liquor and 70-80% are

recycled to a subsequent batch. If whole corn mash is used, however, recycle of yeast is not possible and the spent grain is dried and sold as animal feed.

In one study conducted by Ueda *et al.* (1981) in Brazil, ethanol was produced from raw cassava in a nonconventional fermentation method. A single step method which combined liquefaction, saccharification and yeast fermentation was used without cooking and autoclaving. The reduction of the viscosity of fermentation mashes by commercial glucoamylase and *A. awamori* and *A. niger* Koji enzymes were investigated. Both fungal Koji enzymes contained higher xylan-hydrolysing activity than commercial enzymes which, however, had higher cellulase activity (Ueda *et al.* 1981). Attention has been given to alcohol fermentation without cooking, as a way of saving on the cost of energy consumption. A *Chalara paradoxa* glucoamylase preparation in a mixture of raw corn starch and yeast, was able to yield 63.5 - 86.8% of the theoretical yields by *S. cerevisiae* (Mikuni *et al.* 1987).

The production of ethanol using recombinant DNA technology has received a great deal of interest recently. A yeast strain containing an *Aspergillus* glucoamylase gene was constructed with the ability to produce ethanol from starch (Innis *et al.* 1985). Glucoamylase genes (*STA* 1,2,3) of *Saccharomyces diastaticus* were also cloned in *S. cerevisiae* (Yamashita and Fukui, 1983; Erratt and Nasim, 1989). At the Cetus Corporation, the glucoamylase gene from the mold *Aspergillus* has been cloned into strains of distillers yeast, and integrated into the fermentation process without loss of glucoamylase activity (Finn, 1987). The biggest challenge would be

to produce a yeast strain with the ability to degrade a variety of complex carbohydrates like cellulose and xylose, reducing the overall cost of ethanol production.

1.5.4.1 The Brewing process

Beer can be defined as a malt beverage resulting from an alcoholic fermentation of the aqueous extract of malted barley with hops (Potter, 1986). In the brewing process barley is cleaned and graded according to size. The large grains are left to germinate for a period of about seven days in a process called malting when the natural enzymes present in the grain (amylases and proteases), break down endosperm into fermentable sugars and amino acids. This is followed by a process of kilning where grain is heated for color and flavor development. The malted grain is first passed through a process of coarse milling to form what is called grist. Then follows the process called mashing where the grist is steeped in water at 65°C. At this stage malt adjuncts like starches or sugar may be added. It is also at this stage that additional enzymes amylases and glucoamylases are added to degrade starch into simple sugars which can be completely fermented by brewer's yeast (Broderick, 1977; Lewis and Young, 1995).

The mixture is filtered and spent grain is removed for possible use as cattle feed. The clear liquid called wort is boiled in a wort kettle for about 1.5 h to inactivate

the enzymes. Further processing involves color development, precipitation of proteins, reduction of pH and off flavors may be distilled. Hops are added for flavor and more liquid adjuncts like sugar solutions or corn syrups may be added to increase the final alcohol content. Trub, the name given to spent hops and other sediments, are then removed by filtration. The wort is cooled in a heat exchanger, then inoculated with brewer's yeast (in this case top-fermenting yeast, *Saccharomyces cerevisiae*). Fermentation takes place at 15-22°C for 3-5 days when ethanol and carbon dioxide are produced. Spent yeast is removed for possible reuse or use as animal feed. Carbon dioxide is removed for addition at a later stage (Potter, 1986; Lewis and Young, 1995).

The beer is racked in wooden or stainless steel casks and left to mature for 2-6 weeks at 0°C. At this stage, secondary fermentation takes place. Yeast, tannins and proteins which have settled out (cold trub) are removed. Caramel color is added to meet specifications. The beer is filtered and pasteurized at 60°C for 6-8 min (71-74°C for 15 sec). This is followed by rapid cooling filtering and aseptic bottling. Commercial enzymes like ß-glucanases, proteases, α-amylase and glucoamylase may be added during mashing, in the fermenter or in the finishing cellars. This is done to improve the brewing process, manufacture new products or improve the stability of the final product. Where possible, brewers prefer to add an enzyme to the mash because it is then inactivated in the boiling stage (Lewis and Young, 1995).

1.5.5 Commercial production of glucoamylases

Glucoamylase production is carried out based on the optimal conditions of growth for the microorganism being used. Thus, pH, temperature, aeration and control of contaminants, are some of the parameters which have to be controlled. Most glucoamylase production is carried out using liquid fermentation or submerged fermentation processes, but because of high level of competitiveness in the industry, specific methods remain confidential. Solid state fermentation techniques have also been employed for production, and this method is gaining renewed interest (Pandey, 1995). Attempts have also been made for glucoamylase production using immobilized cells. Glucoamylases are mainly produced by *Aspergillus* sp., however, solid cultures consisting of wheat bran are used to produce glucoamylase from *Rhizopus* sp. with very high yields.

The presence of transglucosidase which causes polymerization, is undesirable and thus several methods have been used to control its action. Transglucosidase can be removed by selective adsorption and a number of methods have been patented for its removal from glucoamylase solutions (Fogarty, 1983; Pandey, 1995). Transglucosidase has been separated from glucoamylase by selective adsorption on acid clays, the use of negatively charged material such as polyacrylic acid (Sternberg, 1975), and by using cellulosic exchangers (Ayers, 1985). Selection of mutant strains which do not produce transglucosidase is seen as

the most beneficial method of producing transglucosidase-free glucoamylase. Some commercial glucoamylases, their trade names and manufacturing companies are listed in Table 1.2.

In 1987, enzymes used in the ethanol fermentations in the United States, amounted to $0.02 - $0.03 per gallon (Inlow *et al.* 1988). With inflation, the average cost of the use of enzymes today is about $0.05 per gallon of ethanol produced (Novo Nordisk Biochem, N.A. Inc.). World wide production of beer in 1990 was reported at 1.14 billion litres, the largest producer being the United States with 239 x 10^2 megaliters (Lee, 1996). Also, low calorie beer production in 1994 accounted for 34% of the U.S. market (65 million hectolitres, hL) and 14% (2.7 million hL) in Canada. Thus, a reduction in the expenditure for the application of enzymes in brewing, would indeed create a substantial profit margin, if efficient means are found to ferment starch and complex oligosaccharides.

1.5.6 Immobilization of glucoamylase

Work has been done to make the use of glucoamylase more cost effective. One innovation is the use of immobilization. Immobilized glucoamylase has been used in the saccharification of a low (degree of polymerization) DP saccharide from chromatographic separation of HFCS to increase the fructose content (Saha and Zeikus, 1989). It has been used to hydrolyse residual maltose from the alkaline

isomerization of maltose (Walon, 1980). In early work in the development of an immobilized glucoamylase, Dextrozyme^{R} was developed by Novo industries in Denmark as a possible solution to replace soluble glucoamylase in the saccharification of starch to glucose syrup (Rugh *et al.* 1979). This immobilized enzyme proved unsuccessful in achieving the DE value of 94% which was required in glucose syrup production. Lower final DE values were obtained and there was a problem of microbial stability at 55°C. These problems are typical in many glucoamylase immobilization studies. Immobilized glucoamylase has been studied on a pilot scale by Lee *et al.* (1976). Glucoamylase was covalently attached to alkylamine porous silica with glutaraldehyde. The immobilized glucoamylase column was operated for 70 d at 38°C with 30% dextrin feed at flow rates of 250-500 kg/day. The syrup produced by this method was only 1-1.5% lower in glucose than that formed by soluble glucoamylase. There are two serious disadvantages in using glucoamylase immobilized on controlled-pore glass in production of dextrose from corn starch. One is the high cost of the carrier and the other is the dissolution of glass with time (Weetall *et al.* 1979). The development of oxide coated glass has increased durability and half-life, which are important economic advantages, but the cost of the carrier still exceeds the value of the final product.

In 1981, Hartmeier at the Research Division of Boehringer, Germany, patented an immobilized glucoamylase which could be used for oligosaccharide degradation in the brewing industry. He reported that 1 kg of enzyme immobilized on

a matrix was enough to react with wort dextrins at a rate of 1 hl per h. The enzyme remained active for 5000 h. When soluble enzyme was employed, at least 500 DM are required. A change in enzyme conformation, however, was encountered when glucoamylase was immobilized to a solid matrix. Enzyme activity and stability were reduced and optimum conditions for enzyme activity must be maintained (Hartmeier, 1981).

An immobilized glucoamylase reactor for preparing a low calorie beer was also patented by Duncombe *et al.* (1984). A superattenuated low calorie beer was produced by passing fermenting beer through an immobilized glucoamylase reactor having glucoamyalse immobilized on a ceramic monolith. Glucoamyalse was covalently bonded by reacting an aldehydic derivative of glucoamylase with an amino functional group on the internal surfaces of the passages to form an aldimine which is reduced to a secondary amine (Duncombe *et al.* 1984). The reactor can sustain high flow rates of beer containing yeast without plugging or fouling. Beer produced using soluble glucoamylase and beer produced using this immobilized system were shown to have similar low calorie contents at the end of fermentation. However, there was little or no trace of the enzyme in the final beer produced after passing beer through the reactor bed, while the soluble enzyme remained in the final product.

Glucoamylase immobilized on porous silica has also been used in an innovative way to determine starch and total carbohydrate in solution. A flow-

injection system was constructed, comprising an immobilized glucoamylase reactor followed by pulsed amperometric detection of the glucose formed, and this resulted in a 26-fold increase in the detection of soluble starch (Larew *et al.* 1988). This rapid method of total carbohydrate determination has been applied to beer samples. Miller and Sugier (1990) investigated the thermal stability of glucoamylase conjugated with oxidized dextrane T40 as a means of stabilizing the enzyme for application at high temperatures in saccharification. This conjugation, however, did not prevent the enzyme from thermal inactivation. Tate and Lyle were among the first to develop a commercially feasible immobilized glucoamylase (Cheetam, 1985).

Recent trends have looked at the entrapment of cells in beads for the continuous production of enzyme. Immobilized cells of *Aureobasidium pullulans* produced glucoamylase continuously in a fluidized bed reactor (Federici *et al.* 1990). Glucoamylase has been produced using *Aspergillus phoenicus* immobilized in calcium alginate beads (Kuek, 1991). Methods for immobilization of glucoamylase has been reviewed in Saha and Zeikus (1989).

1.6 STRUCTURAL ANALYSIS OF GLUCOAMYLASE

1.6.1 The enzyme active site and important amino acids

Glucoamylase from most microorganisms are found to be composed of two domains, namely, a catalytic domain and the raw starch affinity site. Amino acid

sequences for many glucoamylases have been reported (Svensson *et al.* 1983; Yamashita *et al.* 1985; Ashikari *et al.* 1986; Itoh *et al.* 1987; Dohmen *et al.* 1990; Shibuya *et al.* 1992; Hata *et al.* 1991; Ohnishi *et al.* 1992). Using this information structural models for the catalytic and raw starch binding domains have been identified. Aleshin *et al.* (1992) produced a structural model for the catalytic domain of glucoamylase from *Aspergillus awamori.* The mechanism of binding of glucoamylases from *A. awamori* var. *kawachi* to cyclodextrins and raw starch was described by Goto *et al.* (1994). It was found that an inclusion complex was formed when ß-cyclodextrin (ß-CD) bound to the enzyme and glucoamylase was found to stereospecifically recognize the structure of the secondary OH-side but not the primary OH-side of ß-CD. Also, a Trp^{562} residue was found to contribute to the formation of inclusion complexes (Goto *et al.* 1994a)

The N-terminal portion of *R. oryzae* glucoamylase is thought to be responsible for adsorption to starch and the C-terminal portion to be involved in raw starch degrading activity (Yamashita and Fukui, 1984). Similarly, the C-terminal regions of glucoamyalse from *A. niger* and *Saccharomycopsis fibuligera* seem responsible for introducing starch-degrading activity. This type of sequence is lacking from *Saccharomyces* glucoamylases and thus a different mechanism must be present here for starch binding and oligosaccharide hydrolysis (Vihinen and Mäntsälä, 1989).

The catalytic domain of glucoamylase from *Aspergillus niger* was purified and characterized from glucoamylases G1 and G2 using subtilisin with the aim of preparing a fragment for crystallization studies (Stoffer *et al.* 1993). The glucoamylase G1 of *A. niger* consists of three parts: (1) Ala-1-Thr-440, that contains the catalytic site, (2) Ser-441-Thr-551, a highly O-glycosylated linker segment, and (3) Pro-512-Arg-616, a C-terminal domain responsible for raw starch binding (Svensson *et al.* 1983). Limited proteolysis using subtilisin left the N-terminus intact while cleaving primarily between Val-470 and Ala-471. Glucoamylase G1 and G2 gave the same catalytically active domain which did not seem to be in intimate contact with the starch-binding domain (Stoffer *et al.* 1993).

In *Apergillus niger* the O-glycosylated region plays a minor function in binding to hydrogen-bond ordered starch. The O-glycosylated region is thought to be a semi-rigid rod of a random-coil structure (Williamson *et al.* 1992a,b). This would provide an extended peptide backbone and hence a fixed distance in linking the catalytic and starch binding domains. The catalytic and binding domains of glucoamylase 1 from *A. niger* glucoamylase were studied to see how O-glycosylation linker peptides affect their function. When heated, the catalytic domain unfolds irreversibly while the binding domain unfolds reversibly, being stabilized by O-glycosyl linkers (Neustroev *et al.* 1993; Williamson *et al.* 1992a,b). The thermal stability of the catalytic domain was not affected by the binding domain, however, the catalytic domain increased the melting temperature of the binding domain. The O-

glycosylated region in glucoamylase 1 in the intact protein acts as a semi-rigid rod with the main function of providing a backbone to link the catalytic and binding domains. It did not, however, increase the affinity for starch.

Based on chemical modification studies of *A. niger* glucoamylase, tryptophan residues have been proposed to be essential for enzymatic activity (Rao et al., 1981). One residue is thought to be involved in the binding of the substrate and the other in catalytic activity. Trp-120 is responsible for binding of substrate and might maintain structural integrity necessary for catalysis (Clarke and Svensson, 1984a). A short homologous amino acid sequence precedes both this Trp-120, and Trp-83 which is involved in substrate binding in Taka-amylase A (Clarke and Svensson, 1984b; Vihinen and Mäntsälä, 1989). From model fitting studies, it was speculated that glucoamylase might fold in two domains with the NH_2-terminal domain of alternating α- and ß-structure and the COOH-terminal region dominated by the ß-structure. Glucoamylase may belong to the class of enzymes having α/ß-barrel supersecondary structure, like the endo-α-glucanase, Taka-amylase A and porcine pancreas α-amylase (Clarke and Svensson, 1984b).

Mutant glucoamylase genes were constructed by *in vitro* mutations of the *GLU1* DNA coding for *S. fibuligera* glucoamylase in order to find amino acid residues which are required for glucoamylase activity (Itoh *et al.* 1989). Eighteen mutant proteins were assayed for activity and thermal stability and temperature dependency were assessed. It was revealed that Ala81, Asp89, Trp94, Arg96,

Asp97, and Trp166 were required for wild-type levels of activity and Ala81 and Asp89 were not essential to catalytic activity but they played a role in thermal stability.

A subsite theory for analyzing the substrate affinities of glucoamylases has been developed (Hiromi, 1970; Hiromi *et al.* 1973). The active site of glucoamylase was proposed to consist of several subsites. They are numbered from the binding site of glucose at the nonreducing end and the concept assumes that each unit of substrate (i.e. α-D-glucopyranosyl residues) interacts with an individual subsite with a certain affinity. The subsite structure of different glucoamylases showed a common arrangement with 7 subsites in total and the catalytic site being located between subsite 1 and 2 (Ohnishi, 1990; Fagerström, 1991; Ermer *et al.* 1993). In the glucoamylase from *Rhizopus niveus,* subsites 1 and 2 had affinities A1, -0.48 and A2, 4.96 kcal mol^{-1}. Subsite 1 seemed to show negative or zero for many strains and this can be explained by the binding of the transition state structure of a pyranose ring in a half chair configuration for the hydrolytic reaction (Ohnishi, 1990). Subsite 2 has the highest affinity for oligomeric substrates and glucose, followed by decreasing affinity in going towards subsites 3 to 7 (Fagerström, 1991). Subsite binding energies for glucoamylase from *Clostridium* sp. G0005 were also studied by Ohnishi *et al.* (1992). The binding energy at subsite 1 was negative and this was thought to be responsible for the high enzyme activity with isomaltose. Table 1.3

summarizes some amino acids which are important for catalysis and for the binding of substrate to glucoamylase.

1.6.2 Spectroscopic methods used to elucidate glucoamylase structure

Clarke and Svensson (1984b) conducted preliminary experiments to determine which tryptophanyl residues were important in activity of *Aspergillus niger* glucoamylase G1 and G2. The binding of acarbose, a potent inhibitor, was enough to protect 2 tryptophanyl residues from modification by oxidizing agent N-bromosuccinimide. When acarbose was dissociated from the enzyme, 80% of the initial enzyme activity was retained. Oxidized enzyme derivatives were characterized by difference in UV absorption and by fluorescence spectroscopy, which indicated that 2 residues were essential in the mechanisms of glucoamylase action. One was important in the binding of the substrate while the other was part of the catalytic active center (Clarke and Svensson, 1984b). This was later confirmed in experiments where Trp120 was changed to Phe and the effect of this mutation on binding of oligosaccharides was monitored. Stopped-flow fluorescence spectroscopy was used and steady-state kinetic measurements were estimated (Olsen *et al.* 1993). The Trp120→Phe substitution reduces the fluorescence signal by only 12-20%, indicating that other tryptophanyl residues were important in the conformational change.

In recent years, the crytallography of proteins has revealed details of the participation of aromatic side chains in the binding of sugars. Sugar-protein interactions are measured in solution by spectral shifts caused by a change in the microenvironment of the chromophores. Thus, the type and number of aromatic residues affected and the nature of the perturbation can be assessed. Svensson and Sierkes (1992) used difference spectroscopy and four types of inhibitors to determine the roles of aromatic side chains of glucoamylase from *Aspergillus niger*. Reaction of valienamine-derived inhibitors with glucoamylase caused an unusual change in absorbance from 300 to 310-320nm. This change was concluded to arise with the enzyme subsite 2 was occupied in a transition state type complex. Single mutations at Tyr116→Ala and Trp120→Phe altered the perturbations in the spectra. Maltose and maltotriose were suggested to have different modes of binding as observed by perturbations in the spectra (Svensson and Sierkes, 1992). Glucoamylase of *Aspergillus niger* was found to have two different carbohydrate-binding sites, the active site in the catalytic domain, and the starch binding domain in the C-terminal. Acarbose has high affinity for the active site and low affinity for the starch binding domain while α-, ß- and l-cyclodextrins were the opposite.

Additional information gained using perturbation difference spectroscopy in the study of the role of aromatic side chains of glucoamylase from *Aspergillus niger* is summarized in Table 1.3. Kinetic studies have indicated that seven consecutive glucosyl binding sites are found in the enzyme active site.

The functional roles of Trp337 and Glu632 in glucoamylase from *Clostridium* sp. G0005 were determined by chemical modification, mutagenesis and stopped-flow fluorescence kinetics (Ohnishi *et al.* 1994). Chemical modification by N-bromosuccinimide in the presence or absence of the inhibitor acarbose was carried out, with activity being lost irreversibly in the absence of acarbose. The absorbance change at 280 nm suggested that acarbose protected 2 Trp residues from oxidation. Peptide analysis revealed that Trp337 was an important residue in the catalytic activity. The presteady state kinetics using maltotriose as substrate and mutant strains indicated that Trp337 and Glu632 were crucial in the formation of a third and final intermediate preceding the transition state, in glucoamylase reaction (Ohinishi *et al.* 1994).

1.6.3 X-ray Crystallography as a tool in determining glucoamylase action

The mechanism of glucoamylase action and the three dimensional enzyme structure have been proposed using X-ray crystallography. The crystal structure of glucoamylase from *Aspergillus awamori* var. *X100* has been determined to a resolution of 2.2 Å (Aleshin *et al.* 1992). The enzyme consists of a catalytic domain (residues 1-440), an O-glycosylated domain (residues 441-512) and a starch binding domain (residues 513-516). The complete catalytic domain 2.2 Å crystal structure was determined using a proteolytic fragment of glucoamylase I. Harris *et*

al. (1993) used the crystal structure of the complex of 1-deoxynojirimycin with glucoamylase to estimate which amino acids participate in enzyme action. The chair configuration of this compound in the active site involved Arg45, Asp55, Arg 305, and carbonyl 177.

In addition, a water molecule (water 500) formed a hydrogen bond with Glu400 and the 6-hydroxyl of 1-deoxynojirimicin. This water 500 is at an ideal position (3.3 Å) from the "anomeric" carbon of the inhibitor. From the structural arrangement of functional groups, Glu 179 has been suggested as a catalytic acid and Glu 400, a catalytic base, with water 500 as the attacking nucleophile in oligosaccharide hydrolysis (Harris *et al.* 1993).

The crystal structure of the complex of acarbose with glucoamylase from *Apergillus awamori* var *X100* was determined at a resolution of 2.4 Å as a means of determining the mechanism of enzyme hydrolysis (Aleshin *et al.* 1994). All crystallographic work points to subsite 1 as the main site of substrate-enzyme complexing and the studies with acarbose provide information about glucoamylase interaction with an extended substrate. A significant hydrophobic contact was obtained between Trp120 and the third residue of acarbose which distorts the angle of the glycosidic bond between residues 2 and 3 of acarbose. Also, water 500 forms a H-bond with Glu 400 and the 6-hydroxyl of the valienamine group of the inhibitor, and this molecule is at an ideal distance, 3.7 Å from the "anomeric" carbon of the

inhibitor. Although the key functional groups in the inhibitor-enzyme complex are now clear, the precise mechanism of hydrolysis is still obscure (Harris *et al.* 1993).

1.6.4 The glucoamylase protein subfamily

Coutinho and Reilly (1994) have grouped glucoamylases into five subfamilies of close structural similarities. One subfamily is glucoamylase from the bacterial genus *Clostridium*. Two subfamilies comprising two glucoamylases each, are from yeast genera *Saccharomycopsis* and *Saccharomyces*. Another two subfamilies are from filamentous fungi, one comprising *Aspergillus, Humicola, Neurospora* and *Humiconis* glucoamylases and another comprising a single *Rhizopus* enzyme. Seven of the fungal glucoamylases were found to have a starch binding domain connected to the catalytic domain by an O-glycosylated linker (Williamson *et al.* 1992a,b). Yeast glucoamylases had only a catalytic domain but an O-glycosylated region was found in *Saccharomyces diastaticus* glucoamylase (Yamashita, 1989). The N-terminal region of glucoamylase from *Clostridium* sp. G0005 was thought to be responsible for anchoring the enzyme in the cell membrane prior to proteolytic cleavage for release in the medium (Ohnishi *et al.* 1991). Structural similarities in glucoamylases have been evaluated by Coutinho and Reilly (1994) using hydrophobic cluster analysis. These alignments are thought to have potential in the

modelling and modification of glucoamylase by protein engineering to meet specific needs.

1.7 CASE STUDY - Brewing of low calorie beer

1.7.1 Definition of "Low calorie" or "Lite" beer

The main difference between "Lite" and "normal" beers is that "Lite" beers have a reduced calorific value. The reduction could be anywhere between 15-50%. The main features of "Lite" beer are (1) low original gravity giving a lighter mouthfeel than normal beer, and (2) complete fermentation. The majority of "Lite" beers are produced by greatly lowering residual dextrin content (Lewis and Young, 1995). The dextrins in beer arise because the malt amylases are limited in their action on malt starch. Enzymes capable of hydrolysing glycosidic bonds of dextrin may be added to degrade these dextrins, with the polymers being hydrolysed to sugar which is then fermented by yeast. Enzymes from unkilned or lightly kilned malt may be added during mashing or at the fermentation stage or commercial enzymes from fungi and bacteria may be used.

The calorific content in beer can be expressed by the following equation (Lewis and Young, 1995):-

Calories in 10 centiliters = 4 x % (w/v) solids x 7 x % (w/v) alcohol.

In a typical beer, residual dextrins account for 75% of the solids. Regular beer may have the same alcohol content as low calorie beer, but it contains a large percentage of residual carbohydrates - dextrins and unfermentable sugars.

Since its introduction in 1973, Miller's Lite has become extremely popular in U.S.A. (Brenner, 1980). In 1994 "lite beers" accounted for about 34% of the US market (65 million hL) and 14% (2.7 million hL) in Canada (Lee, 1996). Several methods have been used in the production of "Lite" beers. These include dilution of the regular beer itself with water, the use of dextrose as an adjunct to reduce the residual carbohydrate content of beer, and the addition of glucoamylase to the wort to meet the same end.

1.7.2 Problems with the use of glucoamylase in low calorie beer production.

Saccharomyces cerevisiae, the yeast most commonly used in fermentation, is only capable of fermenting glucose, sucrose, fructose, maltose and maltotriose. Larger sugars and starch cannot be used directly and must therefore be hydrolysed, usually by the addition of enzymes or applying heat before fermentation (Erratt and Nasim, 1989). The problem with addition of glucoamylase in the production of low calorie beer is the difficulty in removing all enzyme activity from the final product. Some glucoamylase finds its way into the final beer which is likely to become

sweeter on standing on supermarket shelves (Brenner, 1980). Fungal glucoamylases which are produced commercially tend to be heat stable.

This situation could be avoided by using a thermolabile glucoamylase. The brewing industry commonly pasteurizes beer and the pasteurization step could be used to inactivate thermolabile glucoamylases. The genetic engineering of a brewer's yeast strain with the ability to secrete a thermolabile glucoamylase would be the ideal solution to this problem. The enzyme secreted would convert starch and residual dextrins to glucose and the yeast would then ferment sugar in brewer's wort into alcohol. This yeast would fulfil a dual purpose and would make low calorie beer production more cost effective, eliminating the need to add commercial enzyme preparations. Yeast strains should also be constructed so that they synthesize significant amounts of "debranching" enzymes to facilitate complete hydrolysis of dextrins. The recombinant strains should be catabolite-derepressed for glucoamylase production.

Distiller's yeast used for commercial ethanol production cannot ferment starch. Thus, the introduction of starch degrading activities in yeast is advantageous because costly starch hydrolysis prior to fermentation could be avoided. The engineered yeast strains produced should be suitable for commercial production, i.e., they should be alcohol tolerant and the enzyme produced should not be inhibited significantly by glucose, the end product. Innovations for the improvement of brewery fermentations have been reviewed by Hammond (1988). Ibragimova *et*

al. (1995) developed a strategy for the construction of industrial strains of distiller's yeast. It included construction of congenic genetically marked haploid strains of hybrid nature, integrative transformation of the haploid strains with a DNA fragment containing an expression vector with the gene to be cloned, and hybridization of the transformants, with the isolation of final industrial homozygous strains. Using this procedure, industrial yeast strains were constructed with improved amylolytic activity. A recombinant strain of *S. cerevisiae* producing α-amylase, glucoamylase and pullulanase was developed for one-step enzymatic hydrolysis of starch (Janse and Pretorius, 1995). The introduction of the pullulanase gene, *PUL1* into a *S. cerevisiae* containing both *STA2* and the α-amylase gene *AMY1*, resulted in 99% assimilation of starch.

Hammond and Bamforth (1993) successfully produced a brewer's yeast strain using the glucoamylase gene of *Aspergillus niger*. The lack of α-1,6 debranching activity in glucoamylases derived form *S. cerevisiae var. diastaticus* is a significant problem which can be overcome by cloning glucoamylase genes from other microorganisms. The gene of glucoamylase from *A. niger* codes for a protein with both α-1,4 and α-1,6 activities. To avoid instability problems, three copies of the gene were integrated into different copies of the yeast cloning vector. Fermentation trials in 100 litre fermenters produced good quality "Lite" beer. In 1995, actual production of a "Lite" beers ("Nutfield Lyte"), produced by a recombinant yeast strain, commenced production in the United Kingdom. This was the first example of

commercial production of a low calorie beer using a recombinant yeast strain and the company BRF International of Surrey, England was responsible for the research involved.

1.7.3 The glucoamylase multigene family

The number of glucoamylase coding genes varies among strains. Three structural genes for glucoamylase have been identified in *Saccharomyces cerevisiae* var. *diastaticus*: the DEX gene family or the STA gene family (Meaden *et al.* 1985; Vihinen and Mäntsälä, 1989). In the 1970s two independent studies of the genes responsible for starch fermentation in *S. cerevisiae* var. *diastaticus* revealed three polymorphic genes. Tamaki (1978) described three genes located on different linkage groups *STA*1, *STA2* and *STA*3, which were responsible for starch fermentation. Also Erratt and Stewart (1978) studied dextrin utilization by *S. cerevisiae* var. *diastaticus*, designating the gene *DEX1.* They later described two other genes, *DEX2* and *DEX* 3, which controlled glucoamylase production. In addition, the *Saccharomyces cerevisiae* sporulation glucoamylase gene, *SGA1* also codes for an enzyme but only during the sporulation phase of life (Pugh *et al.* 1989). Starch utilization in the yeast *S. cerevisiae* var. *diastaticus* depends on the expression of three unlinked genes, *STA1* (chromosome IV), *STA2* (chr. II) and *STA3* (chr. XIV). Each gene encodes one of the extracellular glycosylated

glucoamylase isozymes *GAI*, *GAII* and *GAII*, respectively (Pretorius *et al.* 1991). *SGA1* is homologous to the middle and 3' regions of *STA* genes but lacks the 5' sequence that codes for secretion of the extracellular glucoamylases.

The *STA* genes are repressed by the substance *STA*10 or the *MATa*/*MAT* α-encoded repressor in the diploid form, and also the genes are carbon catabolite repressed by glucose (Pretorius *et al.* 1991). Using the *DEX* system to describe glucoamyalse genes from *S. diastaticus*, *DEX1* is allelic to *STA2*, and *DEX2* is allelic to *STA1*. Since in *S. cerevisiae* the three gene fragments hybridize with *STA1*, it has been suggested that a number of genes could have arisen from the translocation of *STA* genes in the chromosome (Vihinen and Mäntsälä, 1989). The nucleotide sequence of the glucoamylase gene *STA1* was determined by Yamashita *et al.* (1985b).

Aspergillus niger and *Aspergillus awamori* both produce two forms of glucoamylase from only one structural gene. The structural gene for *A. niger* contains four introns, and one intron occurs when the messenger RNA for glucoamylase G2 is formed by removing a 169-bp fragment from mRNA. The gene coding for *A. awamori* glucoamylase has four intervening sequences, and these have to be removed before this gene can be expressed in yeast. In addition, a yeast promoter and termination signals are required. *Rhizopus oryzae* has only one structural gene and its nucleotide sequence shows that the gene contains 4 introns

and a signal sequence for 25 amino acids. The *Rhizopus oryzae* gene has been patented by Ashikari *et al.* (1989).

When known glucoamylase sequences were compared, five homologous segments were found, one of which does not seem to be essential for amylolytic activity since this region is not present in *Saccharomyces* glucoamylases (Vihinen and Mäntsälä, 1989; Ohnishi *et al.* 1992). The most related nucleotide sequences were those of *Rhizopus* and *Aspergillus* species.

1.7.4 Cloning of the glucoamylase gene

A brief summary of the protocol which may be used for genetic engineering of a brewer's yeast strain carrying the *Saccharomyces cerevisiae* var. *diastaticus* glucoamylase gene is summarized in Fig.1.6. The gene coding for glucoamylase is isolated from the microbial cell by extracting genomic DNA and cutting the DNA with a specific restriction enzyme. A vector is needed to introduce the gene into *Saccharomyces cerevisiae*. In many cases a plasmid from a microbial cell is used, for example, the *E. coli*-yeast shuttle vector, YEp13. This plasmid is capable of replicating in *E. coli* and yeast. The plasmid is cleaved with the same restriction enzyme as the chromosomal DNA, so that the ends would match those of the DNA. A ligase is used to link plasmid and DNA and brewer's yeast may then be transformed, accepting the recombinant plasmid. A selection process is followed to

identify recombinant strains with the ability to secrete glucoamylase (Erratt and Nasim, 1989, Lee, 1991b).

Recombinant strains with glucoamylase activity may be identified by several methods. Erratt and Nasim (1989) plated transformant cells on nutrient medium containing 2% (w/v) starch. After protoplasts had regenerated the plates were refrigerated at 4°C for 2-3 days. Halo formation, or a slight zone of clearing around some colonies indicated where starch hydrolysis had occurred. Another method of detection commonly applied was to produce a synthetic labelled oligonucleotide probe deduced from a known glucoamylase amino acid sequence or from consensus regions within the aligned amino acid sequences of other glucoamylases (Ashikari *et al.* 1986; Stone *et al.* 1993). This probe may then be used to perform colony hybridization studies after transformant colonies are transferred to a membrane. A spot on the membrane would indicate a colony carrying DNA with homologous sequences to those used for the probe (Ohnishi *et al.* 1992; Stone *et al.* 1993). A summary of some genes which have been cloned and sequenced, is presented in Table 1.4.

The problems with cloning of the glucoamylase gene in brewer's yeast are that expression level tends to be too low for practical production of ethanol (Saha and Zeikus, 1989). In some cases the recombinant plasmid is unstable and easily lost from the cell. The beer produced using recombinant yeast cells sometimes did not maintain the same quality as normal beer.

1.7.4.1 Cloning of mold glucoamylase genes

Glucoamylases are abundant in many mold strains and a great deal of work has been done to clone fungal glucoamylase genes in *Saccharomyces cerevisiae* in order to develop an industrial strain which can produce ethanol from complex carbohydrates. Glucoamylase encoding genes have been cloned and sequenced from several filamentous fungi including *Aspergillus niger* (Boel *et al.* 1984), *Aspergillus awamori* (Innis *et al.* 1985), *Aspergillus oryzae* (Hata *et al.* 1991), *Aspergillus terreus* (Ventura *et al.* 1995), *Neurospora crassa* (Stone *et al.* 1993), and *Rhizopus oryzae* (Ashikari *et al.* 1985, 1986).

Oligoribonucleotide probes derived from amino acid sequences of peptide fragments of glucoamylase, were used to clone the glucoamylase gene of *Aspergillus oryzae* (Hata *et al.* 1991). This strain is very important in the food industry for the production of sake, soy sauce, as well as miso and vast quantities of hydrolytic enzymes are secreted during fermentation. Thus, the expression of the glucoamylase gene of *A. oryzae* in *S. cerevisiae* is of great interest for food industry applications. Ventura *et al.* (1995) described the cloning of a glucoamylase *gla1* gene from *A. terreus*. Since glucoamylase from this strain shows high activity at low pHs and high temperatures, these characteristics make it potentially useful in starch processing industries. Also, the high secretory capability of *A. terreus* has sparked

interest in the promoter and signal sequences of the *gla1* gene to design an expression cassette for production of heterologous proteins. Yeast expressing the *Aspergillus awamori* glucoamylase gene was found to be able to perform glycosylation and enzyme secretion (Innis *et al.* 1985). In order for the gene to be expressed, however, a yeast expression vector consisting of a YEp13 autonomously replicating plasmid, the *E. coli* origin of replication, the yeast 2μ origin of replication, the *bla* gene from pBR322, and a yeast *Leu2* structural gene were needed.

The raw starch-degrading ability of glucoamylase from *Rhizopus* sp. has a great potential in being exploited for saccharification. The cloning of *Rhizopus oryzae* glucoamylase gene from a *Rhizopus* genomic DNA library using a synthetic probe, has been described by Ashikari *et al.* (1986). Yeast cells containing the gene construct were able to secrete glucoamylase into medium containing starch, and grow at almost the same rate as in glucose containing medium.

1.7.4.2 Cloning of bacterial glucoamylase genes

The glucoamylase gene from *Clostridium* sp. G0005 was the first bacterial glucoamylase gene to be cloned. However, the use of glucoamylase from this strain would be hampered since safety issues on toxin forming *Clostridium* sp. are critical for the food industry. The advantage of working with bacterial cells, however, is that genetic manipulation is more simple than with eukaryotic cells. Thus it should be

easier to identify structural and mechanistic roles of individual amino acids. Proteins produced by bacteria tend not to be glycosylated, thus making structural analysis less complicated than with eukaryotic enzymes.

The glucoamylase gene from *Lactobacillus amylovorus* was cloned in *E. coli* (James *et al.* 1996). The glucoamylase from recombinant strains had an optimum pH of 6.0 and optimum temperature of 45°C. The properties of both recombinant and wild type enzymes were similar and these enzymes were labile at temperatures above 55°C (James and Lee, 1996, James *et al.* 1996).

1.7.4.3 Cloning and regulation of yeast glucoamylase genes

Saccharomyces cerevisiae and *Saccharomyces cerevisiae* var. *diastaticus* are closely related yeasts, the main difference being the ability of *S. diastaticus* to carry out fermentation on starch, i.e., it secretes glucoamylase. A glucoamylase gene from *Saccharomyces cerevisiae* var. *diastaticus* has been cloned in *S. cerevisiae* where the enzyme is actively secreted. The synthesis of this glucoamylase confers on *S. cerevisiae* the ability to grow on media containing dextrins as the sole carbon source. This acquired ability is of great interest for the production of ethanol from starch derivatives (Mercier and Colonna, 1988). Erratt and Nasim (1986) cloned the glucoamylase gene from *S. cerevisiae* var. *diastaticus* in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Many experiments

have been conducted to understand the regulation of the glucoamylase gene in *Saccharomyces* sp. (Searle and Tubb, 1981; Inui *et al.* 1989; Okimoto *et al.* 1989; Shima *et al.* 1989; Kuchin *et al.* 1993). Gene expression of transformants has been improved by introducing the Suc2 promoter (Cha *et al.* 1992) or inventing new expression vectors with better signal sequences for heterologous gene expression (Maine *et al.* 1991).

Steyn and Pretorius (1991) described the co-expression of *Saccharomyces cerevisiae* var. *diastaticus* glucoamyalse-encoding gene, *STA2*, and a *Bacillus amyloliquefaciens* α-amylase-encoding gene, *AMY*, in *Saccharomyces cerevisiae*. Results indicated that co-expression of these two genes synergistically enhanced starch digestion and this combination may have potential in food industry applications for saccharification. Another attempt at combining enzyme activities was the experiment conducted by Shibuya *et al.* (1992). An α-amylase/glucoamylase fusion gene which was successfully expressed in *Saccharomyces cerevisiae*, was constructed. The aim was to produce a bifunctional protein with α-amylase and glucoamylase activities in order to eliminate the process and period of saccharification. The resulting fusion protein was found to have higher raw-starch-digesting activity than those of the original α-amylase and glucoamylase, and adsorbed onto raw starch like the glucoamylase (Shibuya *et al.* 1992).

The first patent of a procedure for cloning of a glucoamylase gene which produced a thermolabile enzyme, was submitted by Erratt and Nasim (1989). They

cloned the *STA1* gene from *Saccharomyces cerevisiae* var. *diastaticus* using a modified YEp13 plasmid vector and assayed for transformants carrying the glucoamylase gene by plating on starch agar containing bromocresol purple. Zones of clearing appeared around those colonies with starch degrading ability and a transformant producing thermolabile glucoamylase was obtained.

1.8 CONCLUSIONS

Glucoamylases have played a significant role in the food industry in the saccharification of starch for glucose syrup and HFCS production. The use of this enzyme in fermentation processes for methane and alcohol production may be exploited even more if glucoamylases with the ability to hydrolyse a wide range of starch waste material, can be identified. The genes coding for these enzymes could be cloned in distiller's yeasts to prepare value added products from waste material. The recent commercial production of a low calorie beer, "Nutfield Lyte", using a recombinant yeast strain with the ability to ferment maltodextrins is a great step forward in the application of recombinant DNA technology to solve food processing problems. Modern spectroscopic techniques and X-ray crystallography have been quite useful in deciphering enzyme protein structure and the mechanisms of enzyme action. It is through these studies that proteins may be eventually engineered to meet specific needs.

Table 1.1. Properties of some glucoamylases.

Organism	pH Opt.	Temp. Opt. (°C)	Mol.Wt. (d)	Isoel-ectric point	Carb. content (%)	Raw starch digestion	K_m starch	Reference
Aspergillus awamori	4.5	60	83,700 88,000	3.7		+		Yamasaki *et al.* (1977)
Apergillus niger I	4.5-5.0	60	99,000	3.4	17-19		0.023%	Pazur *et al.* (1971)
II	4.5-5.0	60	112,000	4.0	22			
A. niger van *Teighem*	4.7	65	69,810		17		72.5 mM	Ramasesh *et al.* (1982)
	3.5	60	89,130		25		24.8 mM	
A. oryzae I	4.5	60	76,000	5.6		+	13.30 mg/ml	Saha *et al.* (1979)
II	4.5	50	38,000	5.6		0	6.35 mg/ml	
III	4.5	40	38,000	5.6		0	1.11 mg/ml	
A. saitoi	4.5	50	90,000 70,000	3.85	18.8		0.92% 0.42%	Takashi *et al.* (1981)
Clostridium thermohy-drosulfuricum	4-6	75					0.41 mg/ml	Hyun and Zeikus (1985)
Clostridium thermosacch-arolyticum	5.0	70	75,000				18 mg/ml	Specka *et al.* (1991)
Flavobact-erium sp.	5.5-6.5							Bender (1981)
Halobacterium sodomense RD-26	7.5	65	175,000				0.009%	Oren (1983)
Humicola lanuginosa K13/1	6.6	65-70			+			Taylor *et al.* (1978)
Rhizopus sp.I	4.5	40	74,000	8.7	11	+	0.003%	Takahashi *et al.* (1985)
II	4.5-5.0		58,600	8.7	9.3	+		
III	4.5-5.0		61,400	8.8	13.5	+		
Rhizopus niveus	5.5		60,000	8.45	14.9	+		Ohnishi *et al.* (1990)
Saccharomyces diastaticus I			68,000					Yamashita *et al.* (1985)
II			84,000					Pretorius *et al.* (1991)
III			79,000					
Lactobacillus amylovorus	6.0	55	47,000	4.39			10 mg/ml	James and Lee (1996)

Table 1.2. Commerciai glucoamylases.

Organism	Trade name	Company
Aspergillus niger	AMG 300 L	Novo Nordisk Biochem, N.A. Inc. Franklinton, N.C.
Aspergillus niger	Diazyme L-200	Solvay Enzymes, Elkhart, Indiana
Apergillus niger	G-Zyme	Enzyme Biosystems Ltd., New York, N.Y.
Apergillus satoi	Molsin	Seishin Pharmaceutical Co., Japan
Rhizopus niveus	Glucozyme 12	Amano Enzymes U.S.A. Co. Ltd. Lombard, IL.
Rhizopus delemar		Miles Chemical Co., Elkhardt, Indiana, U.S.A.
Rhizopus delemar		Shin-Nihon Kagaku Kogyo Co. Japan
Rhizopus niveus		Seikagaku Kogyo Co. Ltd., Japan

Table 1.3. Important amino acid residues and their role in glucoamylase from *Aspergillus niger* (Adapted from Svensson and Sierks, 1992).

Amino acid	Function	Reference
Trp 120	Crucial for transition-state stabilization Located near subsite 4	Clarke and Svensson (1984a)
Trp212 Trp417 Trp437	All associated with subsite 1	Svensson and Sierks (1992)
Trp170	Interacts specifically with isomaltose	"
Trp116	Binding role Critical for hydrolysis of the glucoside bond in maltose.	"
Asp176	Catalytic base	Svensson *et al.* (1990)
Glu179	General acid	"
Glu180	Critical for substrate binding at subsite 2	"
Asp55	Localized to subsite 1 Catalytic site	Sierks and Svensson (1993)
Tyr306	Binding to subsites 2 & 4	"
Asp309	Linked to subsite 2 Affects affinities of distant subsites	"
Trp337	Catalytic site	Ohnishi *et al.* (1994)
Glu632	Putative catalytic base	"

Table 1.4. Glucoamylases which have been cloned and sequenced.

Source of glucoamylase gene	Host organism	Reference
A. awamori var. *kawachi*	*Saccharomyces cerevisiae*	Goto *et al.* (1994a)
Asperigillus niger	*Saccharomyces cerevisiae*	Innis *et al.* (1985)
Aspergillus oryzae	*Saccharomyces cerevisiae*	Hata *et al.* (1991)
Aspergillus terreus	*Escherichia coli*	Ventura *et al.* (1995)
Rhizopus oryzae	*Saccharomyces cerevisiae*	Ashikari *et al.* (1985, 1986, 1991)
Saccharomyces diastaticus		
STA1	*Saccharomyces cerevisiae*	Erratt and Nasim (1986, 1987, 1989) Yamashita *et al.* (1985)
	Schizosaccharomyces pombe	Erratt and Nasim (1986)
STA2	*Saccharomyces cerevisiae*	Pretorius *et al.* (1986) Kim *et al.* (1994)
STA3	*Saccharomyces cerevisiae*	Yamashita *et al.* (1985)
Saccharomyces cerevisiae SGA	*Saccharomyces pombe*	Yamashita and Fukui (1984)
Saccharomycopsis fibuligera HUT7212	*Saccharomyces cerevisiae*	Yamashita *et al.* (1985a)
Saccharomycopsis fibuligera KZ	*Saccharomyces cerevisiae*	Hostinová *et al.* (1990)
Clostridium sp. G0005	*Escherichia coli*	Ohnishi *et al.* (1992)
Lactobacillus amylovorus	*Escherichia coli*	James *et al.* (1996)

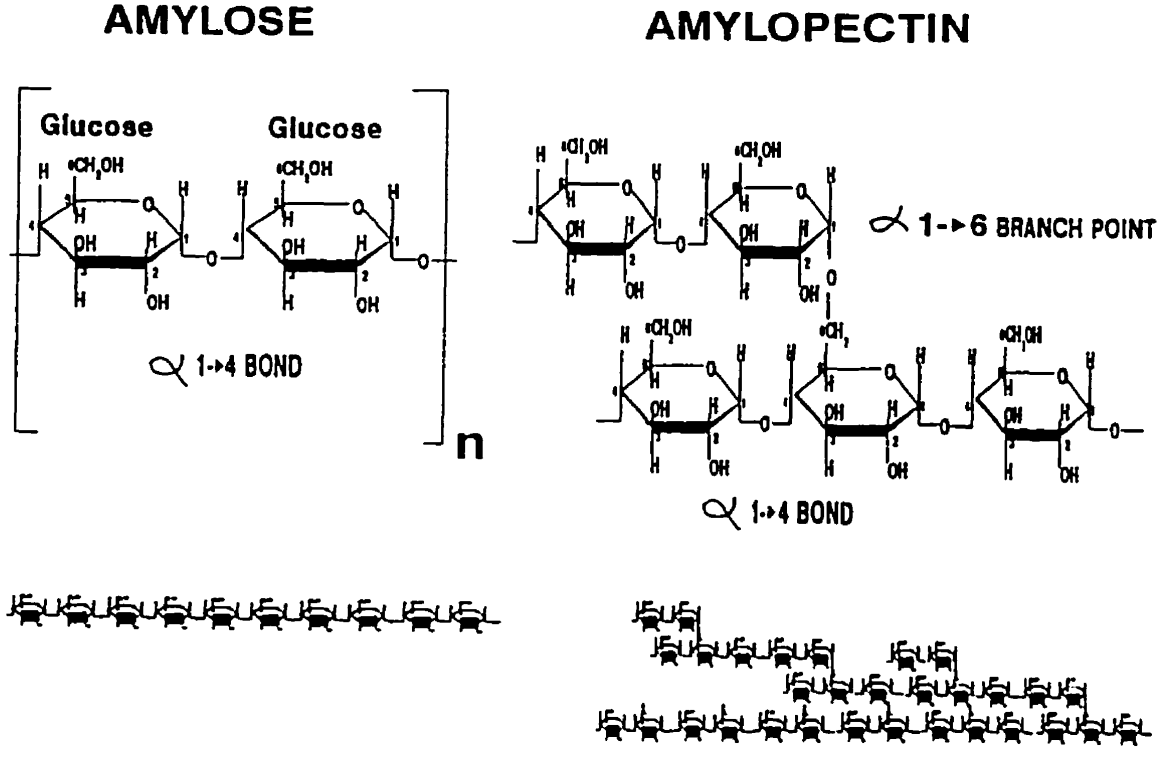


Figure 1.1. Structure of amylose and amylopectin, components of starch.

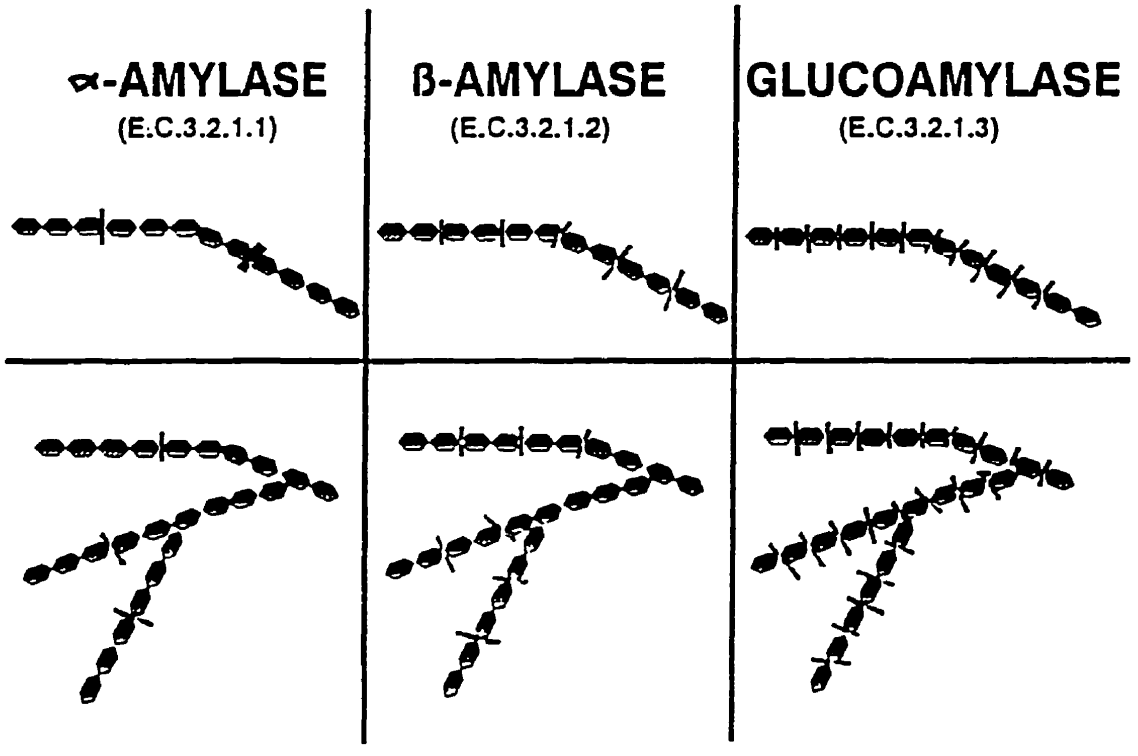


Figure 1.2. Mode of action of carbohydrases. Chains of glucose units are shown, with lines which represent positions of possible cleavage.

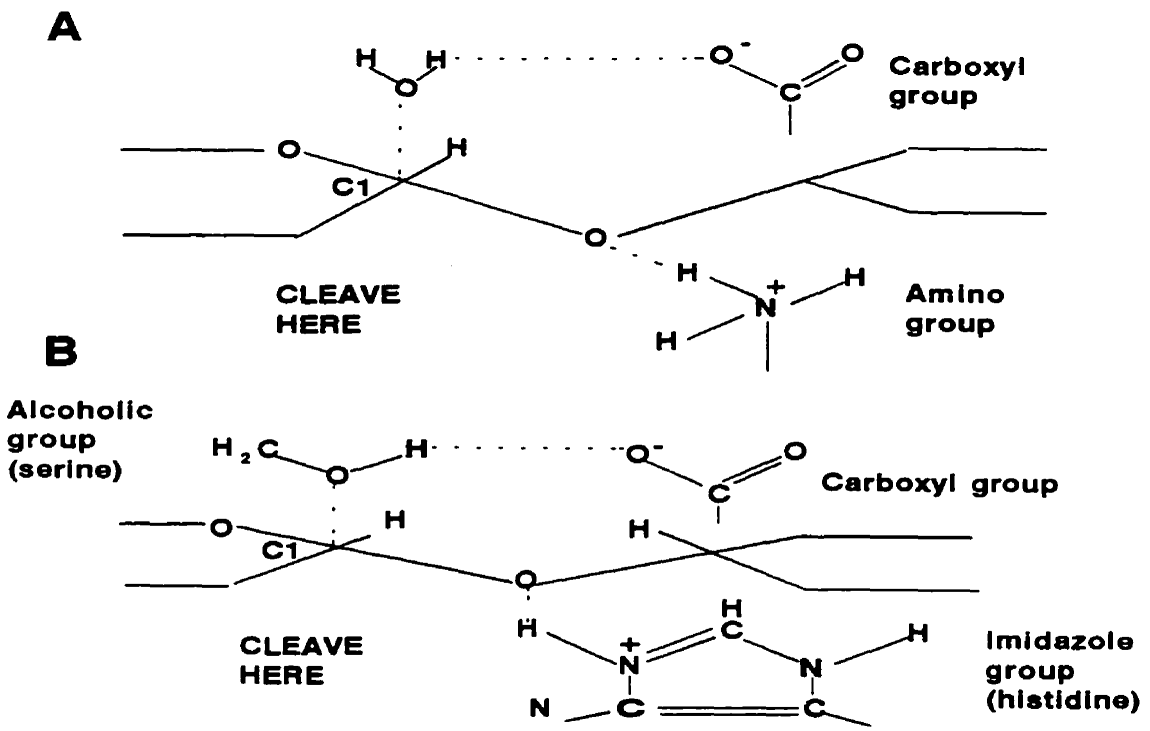
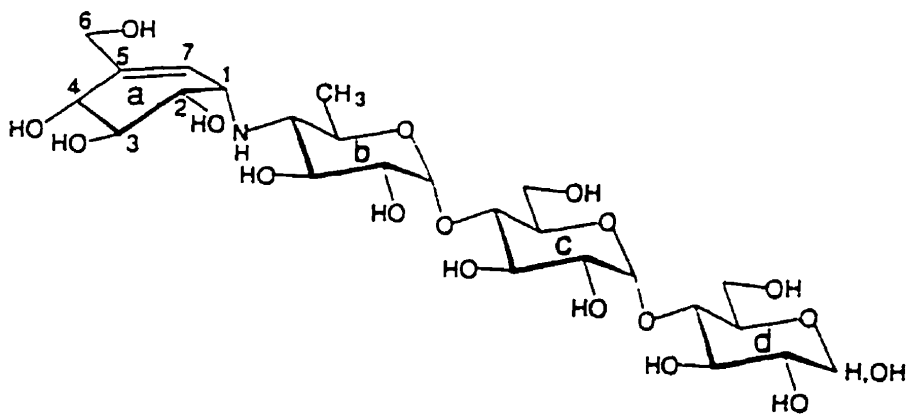
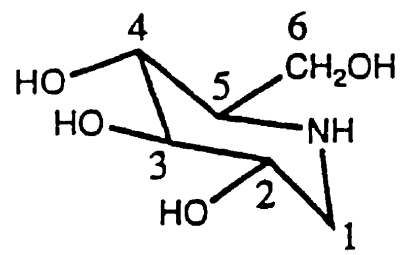


Figure 1.3. Proposed mechanism for glucoamylase action on a glycosidic bond at the enzyme active center (Adapted from Solomon, 1978)



(A)



(B)

Figure 1.4. Structures of glucoamylase inhibitors, (A) acarbose and (B) 1-deoxynojirimycin in the C1-chair configuration (Adapted from Harris *et al.*, 1993; Aleshin *et al.* 1994)

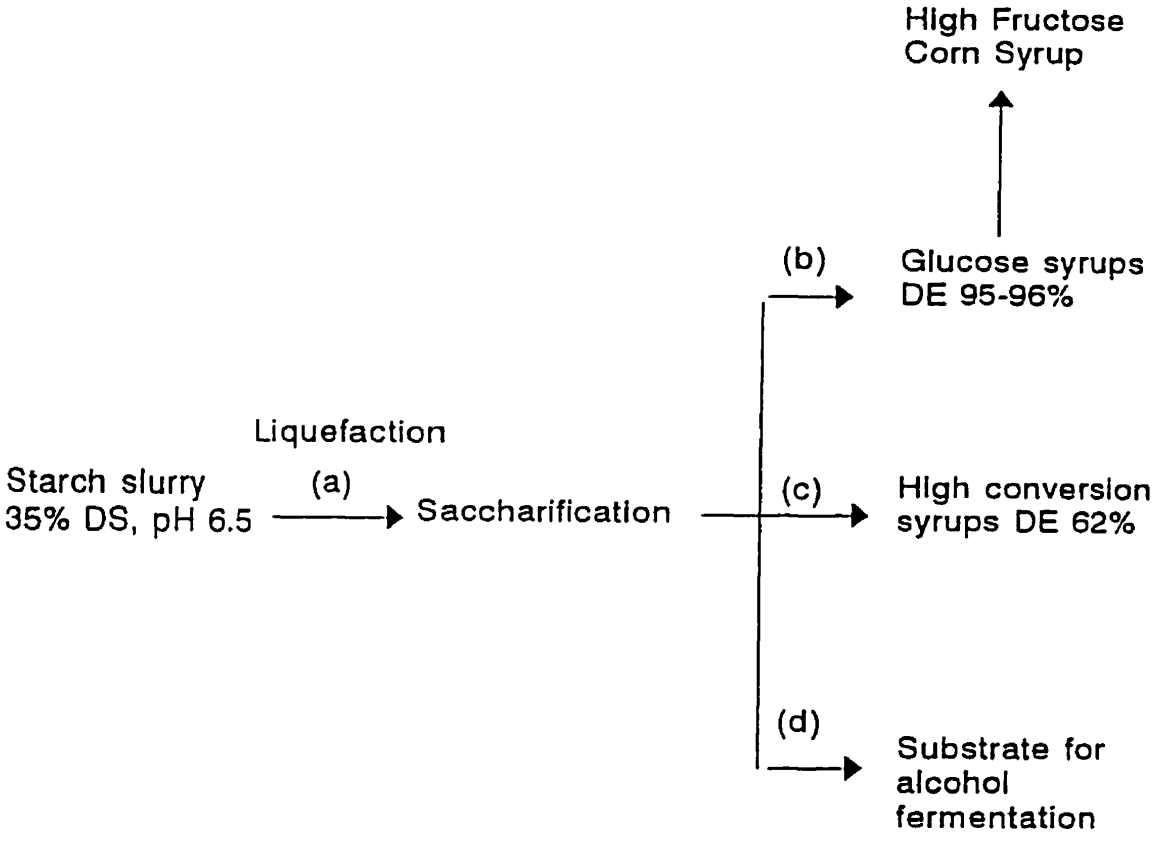


Figure 1.5. Applications of glucoamylase in the processing of starch for glucose production

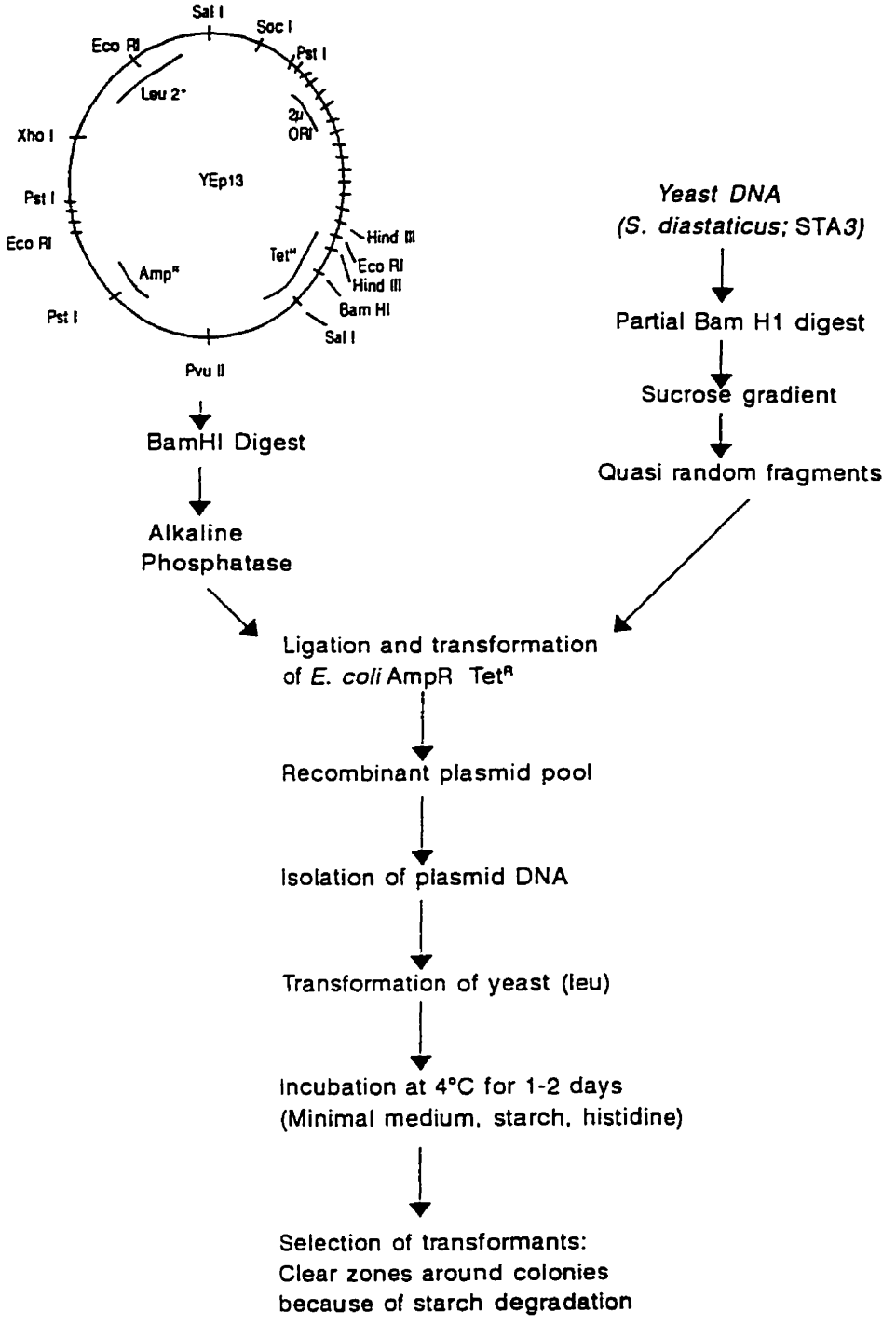


Figure 1.6. A protocol for the genetic engineering of a brewer's yeast strain expressing the *Saccharomyces cerevisiae* var. *diastaticus* glucoamylase (STA3) gene (Adapted from Erratt and Nasim, 1985).

CHAPTER 2

CULTURAL CONDITIONS FOR PRODUCTION OF GLUCOAMYLASE FROM *LACTOBACILLUS AMYLOVORUS* ATCC 33621

In order to establish the potential applications of an enzyme, it must be isolated, purified and characterized. In this chapter we described the isolation of glucoamylase from *Lactobacillus amylovorus*. The conditions for maximal growth and glucoamylase production were established. These conditions were used to produce cell mass for further studies. Protein was prepared from cell mass for the purification and characterization of glucoamylase.

The major results of this study were summarized and submitted as a manuscript for publication in the Journal of Applied Bacteriology. The manuscript entitled "Cultural conditions for production of glucoamylase from *Lactobacillus amylovorus* ATCC 33621" was co-authored by Jennylynd A. James and Byong H. Lee. The project was supervised by Dr. Byong Lee, while the actual experimental work, and writing of the manuscript were done by Jennylynd James. Dr. Byong Lee edited the manuscript prior to its being submitted for publication.

2.1 ABSTRACT

Lactobacillus amylovorus ATCC 33621 is an actively amylolytic bacterial

strain which produces a cell bound glucoamylase (E.C.3.2.1.3.). Conditions of growth and glucoamylase production were investigated using dextrose free de Man Rogosa Sharpe (MRS) medium in a 1.5 L fermenter, with varying dextrin concentration (0.1%-1.5% (w/v)), pH (4.5-6.5), and temperature (25°C-55°C). Cell extracts were prepared by subjecting cells to treatment with a French Pressure cell in order to release intracellular proteins. Glucoamylase activity was then assayed. The effects of pH (4.0-9.0), temperature (15°C-85°C) and substrate (dextrin and starch, 0%-2% w/v) concentration on crude enzyme activity were investigated. Optimal growth was obtained in MRS medium containing 1% (w/v) dextrin, at pH 5.5 and 37°C. Glucoamylase production was maximal at the late logarithmic phase of growth, during 16-18 h. Crude enzyme had a pH optimum of 6.0 and temperature optimum of 55°C. With starch as the substrate, maximal activity was obtained at a concentration of 1.5% (w/v). The effects of ions and inhibitors on glucoamylase activity were also investigated. Enzyme activity was not significantly influenced by Ca^{2+} and EDTA at 1 mM concentration, however Pb^{2+} and Co^{2+} were found to inhibit the activity at concentrations of 1 mM. The crude enzyme was found to be thermolabile when glucoamylase activity decreased after about ten minutes exposure at 60°C. This property can be exploited in the brewing of low calorie beers where only mild pasteurization treatments are used to inactivate enzymes. The elimination of residual enzyme effect would prevent further maltodextrin degradation and sweetening during longterm storage, thus helping to stabilize the flavour of beer.

2.2 INTRODUCTION

Glucoamylase [(1→4)-α-D-glucan glucohydrolase, amyloglucosidase, EC 3.2.1.3] is an exo-acting enzyme that yields ß-D-glucose from the non-reducing chain-ends of amylose, amylopectin and glycogen by hydrolysing α-1,4 linkages in a consecutive manner (Fogarty, 1983). The α-1,6 glycosidic bonds are also hydrolysed, but at a much reduced rate.

This saccharifying ability has made glucoamylases important in the commercial production of glucose syrups from starch. Glucoamylases have been applied to the brewing industry, dextrose production, the baking industry and animal feed industry (Fogarty and Kelly, 1980; Fogarty, 1983). The applications, properties and reaction kinetics of glucoamylase have been reviewed by several researchers (Matsumura et al. 1988; Saha and Zeikus, 1989; Vihinen and Mäntsälä, 1989).

The typical enzyme of choice in the food industry is the thermostable enzyme obtained from fungi, since this permits rapid reactions at high temperatures. Mold strains which have been known to produce this enzyme include *Aspergillus awamori* (Flor and Hayashida, 1983; Vialta and Bonatelli, 1988), *A. niger* (Pazur et al. 1971; Pandey and Radhakrishnan, 1993), *A. terreus* (Ghosh et al. 1990; Ali and Hossain, 1991), *Rhizopus oryzae* (Ashikari et al. 1989; Yu and Hang, 1991), *R. niveus* and *R. delemar* (Ohnishi, 1990; Ohnishi et al. 1990). Glucoamylase has also been demonstrated in sporulating yeast strains of

Saccharomyces cerevisiae (Kleinman et al. 1988; Pugh et al. 1989), *S. diastaticus* (Lambrechts et al. 1991) and *S. fibuligera* (Itoh et al. 1989). Many of these strains also produce transglucosidase which affects the efficiency of saccharification with the formation of oligosaccharides from glucose. *Rhizopus* sp., however, do not generally produce transglucosidase, therefore they have been widely used in the Amylo-process industry for conversion of starch to sugar with high efficiency.

In low calorie beer production, there is a need for a thermolabile glucoamylase which can be more readily inactivated by mild pasteurization. The enzymes obtained from molds, at present, to breakdown maltodextrins into simple sugars for yeast fermentation, tend to be thermostable, with residual activity lingering after production. However, the product tends to become sweeter on storage as the thermostable glucoamylase exhibits continual activity. This is undesirable since the product taste and flavour become altered. Another microbial source of glucoamylase, or the cloning of the microbial gene coding for glucoamylase into Brewer's yeast (*Saccharomyces cerevisiae*) would be of great benefit, eliminating the addition of commercially prepared enzymes and reducing overall production costs.

As an alternative source of glucoamylase, bacteria would provide the advantage of a short generation time: 24 h versus three to five days for mold cultivation. Unlike amylases and pullulanases, glucoamylases are rare in prokaryotes. Glucoamylase activity has been demonstrated in a few aerobic bacteria such as *Bacillus stearothermophilus, Flavobacterium sp.* and

Halobacterium sodomense (Bender, 1981; Oren, 1983) and the anaerobes *Clostridium acetobutylicum and C. thermohydrosulfuricum* as well as other *Clostridium* sp. (Hyun and Zeikus, 1985; Chojecki and Blaschek, 1986; Specka et al. 1991; Ohnishi et al. 1991; 1992). Enzymes from *Clostridium sp.* are not generally regarded as safe (GRAS) by the food industry since they produce toxins. Moreover, certain *Bacillus* sp. have been known to transmit foodborne infections capable of causing human diseases, so enzymes from this genus are not readily accepted in the food industry.

One group of prokaryotes, lactobacilli, are generally regarded as safe (GRAS) and have found many applications in the food fermentation industry. Since lactobacilli are microaerophilic, they would not require complex aeration schemes for large scale production in fermenters, like molds. Of special interest is *Lactobacillus amylovorus*, first isolated from cattle waste-corn fermentations (Nakamura, 1981). This species secretes large quantities of α-amylase (Burgess-Cassler and Imam, 1991; Imam et al. 1991; Pompeyo et al. 1993) explaining its ability to rapidly digest corn-starch. *Lb. amylovorus* has also been found to produce other amylolytic enzymes which have not yet been identified or characterized (Fitzsimons and O'Connell, 1994). Its active amylolytic ability makes this strain a good candidate for the investigation of glucoamylase production and for ascertaining future applications in the food industry.

We undertook the present study to determine the location of glucoamylase activity in *Lb. amylovorus* and optimize conditions of growth and glucoamylase

production by investigating effects of temperature and pH. From preliminary experiments, glucoamylase production was found to be inhibited by the presence glucose in the growth medium. This may be explained by product inhibition, which is observed in glucoamylase production from mold strains. Growth and glucoamylase production using the complex carbon source, dextrin, was investigated. Moreover, studies were conducted to investigate characteristics of the crude enzyme by looking at effects of pH, temperature, substrate concentration, storage temperature, ions and inhibitors, on enzyme activity. The enzyme was not particularly thermostable and thus may be suitable for application in the brewing industry. The final stage in brewing is pasteurization at 60°C-65°C for a few minutes before bottling. These temperatures would be adequate to inactivate glucoamylase from *Lb. amylovorus.*

2.3 MATERIALS AND METHODS

2.3.1 Organism and growth conditions

Lactobacillus amylovorus ATCC 33621 obtained from American Type Culture Collection (Maryland, U.S.A.) was activated by two successive transfers in MRS (de Man et al. 1960) broth (Difco Laboratories, Detroit, MI.) and maintained in a sterile broth/glycerol (60%:40%, v/v) solution at -40°C. Working

cultures were prepared by two successive transfers of stock culture to MRS broth at 18 h intervals at 37°C.

Growth experiments were conducted in MRS medium without dextrose (Institut Rosell Inc., Montreal, Canada), in a closed 1.5 L fermenter (BIOSTAT M, Braun, F.R.G.). The medium and fermenter were sterilized in an autoclave at 121°C for 15 min. The strain was cultivated under microaerophilic conditions which were achieved by a small headspace above the medium in a closed fermenter, and with minimal agitation (70 r.p.m.) during growth. Cell culture (0.1%, v/v) was inoculated and incubated at the varying conditions described below, for 28 h.

Optimal growth conditions of the strain were determined by varying concentrations (0.1%, 0.5%, 1.0% and 1.5% w/v) of potato dextrin (Sigma, St. Louis, Mo.) in basal MRS broth. Three trials were conducted at 30°C and the pH was not controlled. Trials were then conducted to determine optimal pH and optimal temperature for growth and glucoamylase production. Firstly, cells were cultivated in medium with the pH controlled using 0.1 M HCl (pH 4.5, 5.5, 6.5) and with temperature fixed at 30°C and dextrin concentration 1% (w/v). Then, the optimal temperature of growth and glucoamylase production was investigated at a fixed substrate concentration (1%, w/v dextrin), pH 5.5 and varying temperature, 25°C, 30°C, 37°C, 45°C and 55°C, during cell cultivation.

Growth curves of *Lb. amylovorus* were prepared from 100 ml aliquots taken at 4-6 h intervals during the growth cycle of each experimental run. A 1 ml portion of sample withdrawn at each time interval was serially diluted in 0.1% peptone. A

volume of 0.1 ml of an appropriate dilution was spread on MRS agar plates in triplicates and incubated at 35°C for 48 h. The colonies which appeared on the MRS agar plates were counted and expressed as colony forming units per ml (cfu/ml). The optical density during cell growth was measured at 600 nm (Beckman DU-64 Spectrophotometer). Intracellular glucoamylase activity of cells in these 100 ml aliquots was also determined as outlined below.

2.3.2 Preparation of cell extracts for glucoamylase localization

In order to isolate cell bound glucoamylase, *Lb. amylovorus* cells were removed from cell culture by centrifugation (Beckman centrifuge, J2-21, Palo Alto, California) at 10,000 g for 15 min at 4°C. Glucoamylase activity in the resulting supernatant was measured. The cell pellet was then washed twice with 0.05 M sodium phosphate buffer (pH 7.0) to remove culture broth. The washed cells were suspended in 10 ml of the same buffer and the cell extract prepared by disrupting suspended cells using a French Press (S.L.M. American Instruments Company, Inc., Urbana, Illinois.), maintained at 80 MPa. Cell debris was removed by centrifugation at 15,000 g for 35 min at 4°C and the supernatant was used to determine intracellular glucoamylase activity. The resultant pellet was then washed in 5 ml phosphate buffer (0.05 M, pH 7.0) to remove any glucoamylase trapped in the solution with cell debris. The suspension was centrifuged at 18,000 g for 35 min at 4°C and the supernatant combined with the original cell extract. The pellet

was resuspended again in 5 ml 0.05 M phosphate buffer (pH 7.0) and the membrane bound enzyme activity determined. The procedure is outlined in Fig. 2.1.

2.3.3 Glucoamylase assay

Glucoamylase activity was determined by the method of Ghosh et al. (1990) with modifications. A 100 µl aliquot of cell extract was incubated with 100 µl 1% (w/v) soluble starch (Sigma) in 0.05 M sodium phosphate buffer pH 6.0 at 45°C for 30 min. The reaction was terminated by immersing the tube in a boiling water bath for 5 min. The glucose liberated by enzymatic action was measured by a glucose oxidase method (Glucose-Trinder test kit, Sigma), using glucose standards (Sigma) for calibration of a standard curve (Fig. 2.2). One unit of glucoamylase was defined as the amount of enzyme liberating 1 µmol/l of glucose per min under assay conditions. Specific activity was defined as enzyme units per mg protein.

2.3.4 Protein assay

Protein was determined spectrophotometrically by the Bicinchoninic Acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL), (Smith et al. 1985). The method is based on the reaction of Cu^{2+} with peptides in alkaline solution to form Cu^{+} ions which are detected by a highly sensitive and selective

BCA reagent. A 0.1 ml sample of enzyme solution was added to 2.0 ml of the assay mixture which was incubated at 37°C for 30 min. The absorbance was measured at 562 nm. Bovine serum albumin (Pierce) was used to prepare the standard curve (Fig.2.3).

2.3.5 Properties of the crude enzyme

2.3.5.1 Effect of pH and temperature

Cells grown at pH 6.0, 37°C and 1% (w/v) dextrin concentration were harvested at the late logarithmic phase of growth (18 h). Cell extract was prepared and the effect of pH on activity of intracellular enzyme was measured in 0.05 M sodium citrate buffer, pH 4.0 to 6.0, 0.05 M sodium phosphate buffer, pH 6.0 to 8.0, and 0.05 M Tris(hydroxymethyl)-aminomethane (Tris) buffer pH 9.0. Cell extract (100 µl) was incubated with substrate (100 µl of 1% (w/v) starch solution in the appropriate pH buffer) for 30 min at 45°C. The glucose released was measured using the glucose oxidase method. The experiment was conducted in triplicate.

The effect of temperature on glucoamylase activity was measured in the range of 15°C-85°C. A solution of 1% (w/v) starch in 0.05 M sodium phosphate buffer, at pH 6.0, was equilibrated for 2 min at the test temperature before an equal volume of crude enzyme was added. The mixture was then incubated for 30

min at the test temperature and enzyme activity assayed by measuring the amount of glucose liberated. Stability of glucoamylase at varying storage temperatures was estimated by incubating crude extract at different temperatures (4°C, 20°C, 25°C, 30°C, 40°C, 50°C, 55°C, 60°C) and sampling the heat treated enzyme at ten minute intervals for 2 h assaying for residual glucoamylase activity. All figures were computer generated with the production of best fit curves (Software Publishing Corporation, 1991).

2.3.5.2 Substrate specificity

The effect of carbon sources, starch (Sigma) and potato dextrin (Sigma) on enzyme activity was determined by varying substrate concentrations (0.1%, 0.5%, 1.0%, 1.5% and 2.0%, w/v). Substrate was dissolved in 0.05 M sodium phosphate buffer at pH 6.0 and made up to the appropriate weight/volume concentration. A glucoamylase assay was then conducted to determine the amount of glucose liberated.

2.3.5.3 Inhibition and metal ion effect

Crude cell extract was incubated in the presence of cations (Cu^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, K^{+}, Ca^{2+}) at final concentrations of 0.1 mM and 1.0 mM, and inhibitors [Ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT),

phenylmethylsulphonylfluoride (PMSF)] at final concentrations of 0.1 mM and 1 mM in 0.05 M sodium phosphate buffer (pH 6.0), for 5 min at 37°C . The reaction was initiated by adding an equal volume of 1% (w/v) soluble starch in 0.05 M phosphate buffer (pH 6.0) and glucoamylase activity was assayed. Inhibition was expressed as a percentage of the activity without effector (control).

2.4 RESULTS

Glucoamylase activity was produced intracellularly when dextrin was used as a carbon source in the growth medium. Negligible activity was observed in the supernatant from *Lb. amylovorus* ATCC 33621, while most of the enzyme activity was detected in cell extract. Fig. 2.1 gives the localization of glucoamylase activity in 1 L of culture broth. About 70% of glucoamylase activity in 1 L culture, containing 7 g of cells, was found to be intracellular. We therefore decided to use cell extract for glucoamylase for further studies.

Optimal growth and glucoamylase production of *Lb. amylovorus* was obtained in MRS medium containing 1% (w/v) dextrin at 30°C, during the late logarithmic phase of growth (16-18 h; Fig. 2.4). At a fixed substrate concentration (1 % (w/v) dextrin in basal MRS) growth and glucoamylase production of *Lb. amylovorus* were found to be optimal at pH 5.5 (Fig. 2.5). Fig. 2.6 shows that maximal growth was obtained at 45°C with a similar pattern of growth being observed at 37°C, however cells died more rapidly at 45°C than at 37°C. Cells

remained alive at 25°C but no growth was observed while cells died rapidly at 55°C. Enzyme production was maximal at 45°C during the period of 16-18 h of growth. This was closely matched with enzyme production at 37°C.

Optimum pH and temperature for glucoamylase activity of the crude extract were pH 6.0 and 55°C, respectively (Table 2.1). The effect of substrate concentration on enzyme activity was determined by varying soluble starch and potato dextrin concentrations (0.1%, 0.5%, 1.0%, 1.5%, 2.0%) at the optimal enzyme conditions previously determined (pH 6.0 and 60°C; Table 2.2). When starch was used as a substrate, maximal activity was obtained at 1.5% starch (w/v). The production of glucose increased linearly as dextrin concentration was increased up to 2% dextrin (w/v). The effects of various monovalent and divalent ions were studied. Amongst the metal ions tested, Ca^{2+}, Mg^{2+}, Mn^{2+} had little effect on enzyme activity at 1.0 mM, however, Pb^{2+}, Cu^{2+} and Co^{2+} were found to inhibit activity at 1.0 mM (Table 2.3). The metal chelator, EDTA, did not significantly inhibit enzyme activity at a final concentration of 1 mM. In stability studies of the crude enzyme, glucoamylase was found to be stable at refrigeration temperatures (4°C-7°C) for up to three weeks. The enzyme also remained stable at room temperature (≈25°C) for up to 24 h. No loss of glucoamylase activity was observed between 40°C and 50°C within a 2 h interval, however activity was lost at 60°C.

2.5 DISCUSSION

Glucoamylase production was found to be induced by the presence of dextrin in the growth medium. When dextrins enter cells they have to be broken down to glucose for further metabolism. This process may explain the enhanced glucoamylase production found intracellularly. The results correlate with observations made by Burgess-Cassler (1993) who found that α-amylases (160 kd and 140 kd), which were secreted into the culture medium by *Lb. amylovorus*, were enhanced by the use of cyclodextrin as the carbon source for growth.

Lb. amylovorus, a Gram positive, non-sporeforming, rod is known to grow at 45°C, actively fermenting starch with the production of extracellular amylolytic enzyme activity (Nakamura, 1981). Thus, in studying the effect of temperature on growth and glucoamylase production, elevated temperature (37°C, 45°C) was found to correlate with an increase in cell mass and enzyme production.

Maximal growth and glucoamylase production in *Lb. amylovorus* ATCC 33621 were obtained after 16-18 h of growth when cells were cultivated in MRS medium supplemented with 1% (w/v) dextrin, at 45°C and pH 5.5. This was the optimal time to harvest cells and isolate glucoamylase for further studies. Optimal glucoamylase activity was obtained at pH 6.0, 60°C and 1.5% (w/v) starch concentration. This optimal temperature would prove favourable in food processing operations like the brewing of low calorie beer, where pasteurization temperatures may be used to inactivate the enzyme after fermentation. Since EDTA was not

found to significantly affect enzyme activity at 1 mM, it is possible that no metal ion is required for enzyme action.

Enzyme activity was compared with starch and potato dextrin to determine which substrate would be more readily broken down. It was found that dextrin, the less complex of the two, was more readily digested. In preliminary growth studies, a greater concentration of dextrin (w/v) could be dissolved in the growth medium, giving equivalent growth of *Lb. amylovorus* as in starch containing media. It would thus seem more feasible to use dextrin in applied fermentations, since this compound also stimulates glucoamylase production.

Thus, *Lb. amylovorus* glucoamylase is active at 40-60°C with an ideal optimal pH (6.0) that might be suitable for the hydrolysis of maltodextrin. The expensive process of isolating an intracellular enzyme does not make it commercially competitive with glucoamylase of mold strains used. However, glucoamylase from *Lb. amylovorus* has the advantage of being sensitive to ordinary pasteurization temperatures. This would reduce the risk of residual enzyme activity remaining in the finished product, causing sweetening and off-flavours. The industrial benefits would include increased shelf life without a change in flavour.

Table 2.1. Effects of temperature and pH on glucoamylase activity of *Lactobacillus amylovorus*.

pH	Specific activity (Units/mg protein)	Temperature (°C)*	Specific activity (Units/mg protein)
3	9.86 ± 0.69	25	0.03 ± 0.00
4	14.44 ± 1.94	40	9.44 ± 0.41
5	24.82 ± 1.95	45	13.14 ± 0.35
6	43.89 ± 3.19	55	13.97 ± 0.22
7	33.43 ± 2.02	60	11.12 ± 0.44
8	19.32 ± 1.33	70	7.50 ± 0.11
9	24.00 ± 0.86		

*Determination of the temperature optimum was performed with crude extract in 0.05 M sodium phosphate buffer at pH 6.0.

Table 2.2. Effect of substrate concentration on the crude glucoamylase activity.

Concentration (%) w/v	Specific activity (Units/mg protein) ± SD	
	Dextrin	Starch
0.25	10.76 ± 0.73	13.87 ± 0.74
0.50	21.73 ± 0.37	23.33 ± 0.18
0.75	30.77 ± 0.46	26.26 ± 0.49
1.00	42.75 ± 0.37	36.44 ± 0.31
1.25	42.38 ± 1.27	37.77 ± 0.55
1.50	50.88 ± 0.57	47.77 ± 0.56
1.75	56.64 ± 2.08	40.42 ± 0.46
2.00	61.48 ± 0.49	37.62 ± 0.92

Table 2.3. Effects of metal ions and inhibitors on the activity of crude enzyme from *Lactobacillus amylovorus*.

Ion/Inhibitor	Relative activity (%) ± SD	
	0.1 mM	1.0 mM
Ca^{2+}	105 ± 0.21	98 ± 1.02
Mn^{2+}	93 ± 0.21	106 ± 0.65
Mg^{2+}	106 ± 0.88	103 ± 0.65
Pb^{2+}	108 ± 0.21	74 ± 3.42
Cu^{2+}	98 ± 2.23	57 ± 0.24
Co^{2+}	99 ± 1.50	57 ± 0.47
K^{+}	97 ± 7.73	85 ± 0.27
EDTA	94 ± 4.51	80 ± 2.36
DTT	100 ± 3.22	82 ± 1.29
Iodoacetate	100 ± 3.00	86 ± 2.57
PMSF	103 ± 0.43	86 ± 3.01

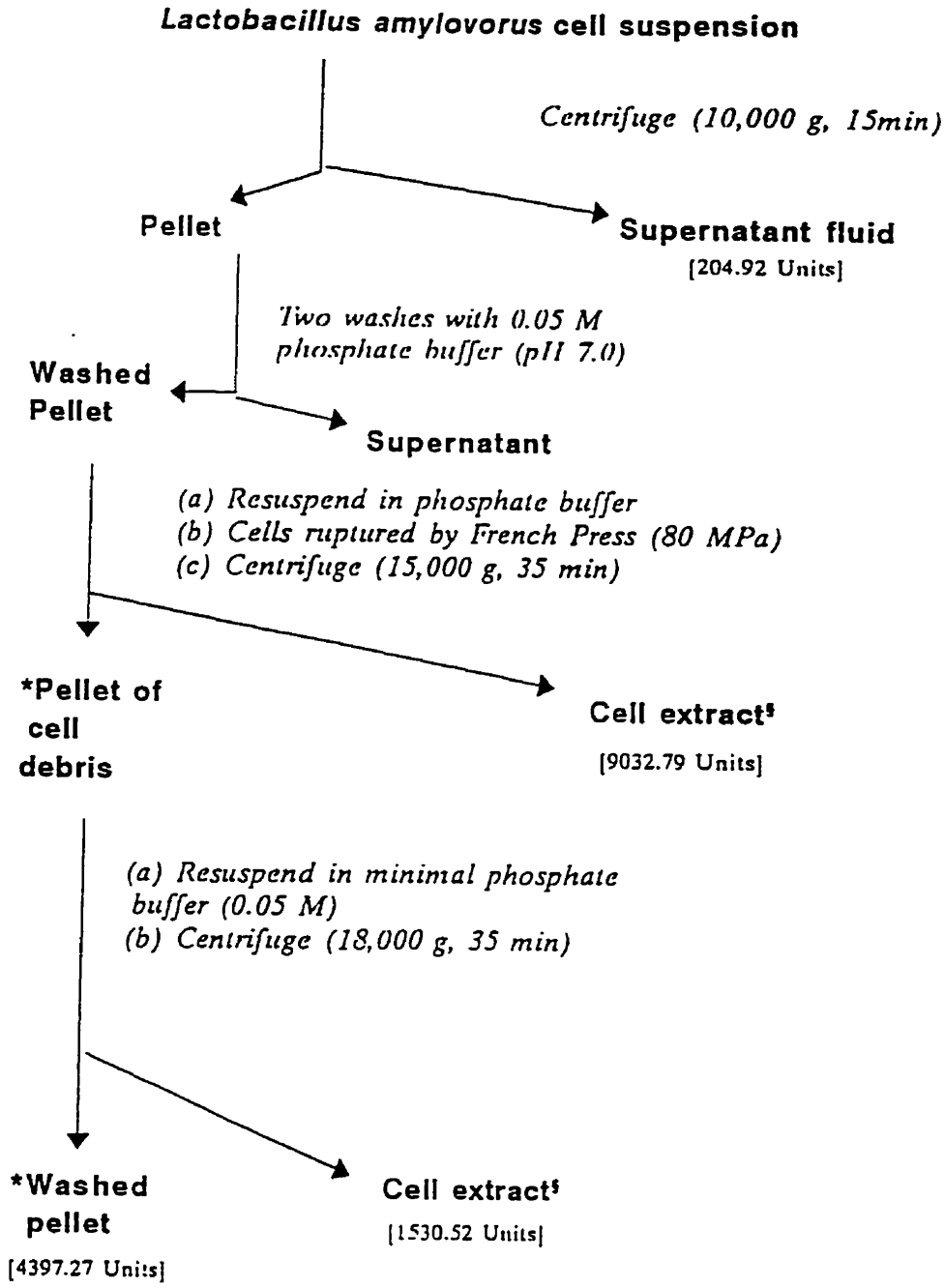


Figure 2.1. Localization of glucoamylase activity in *Lactobacillus amylovorus*, from 1 L of cell culture grown with 1% (w/v) dextrin. Enzyme activity was assayed using starch as substrate in 0.05 M phosphate buffer (pH 6.0). Glucose liberated by enzyme action was measured using a glucose oxidase method. The units of activity given, represent an average of three trials (± 100 units). *Membrane-bound activity; §intracellular activity.

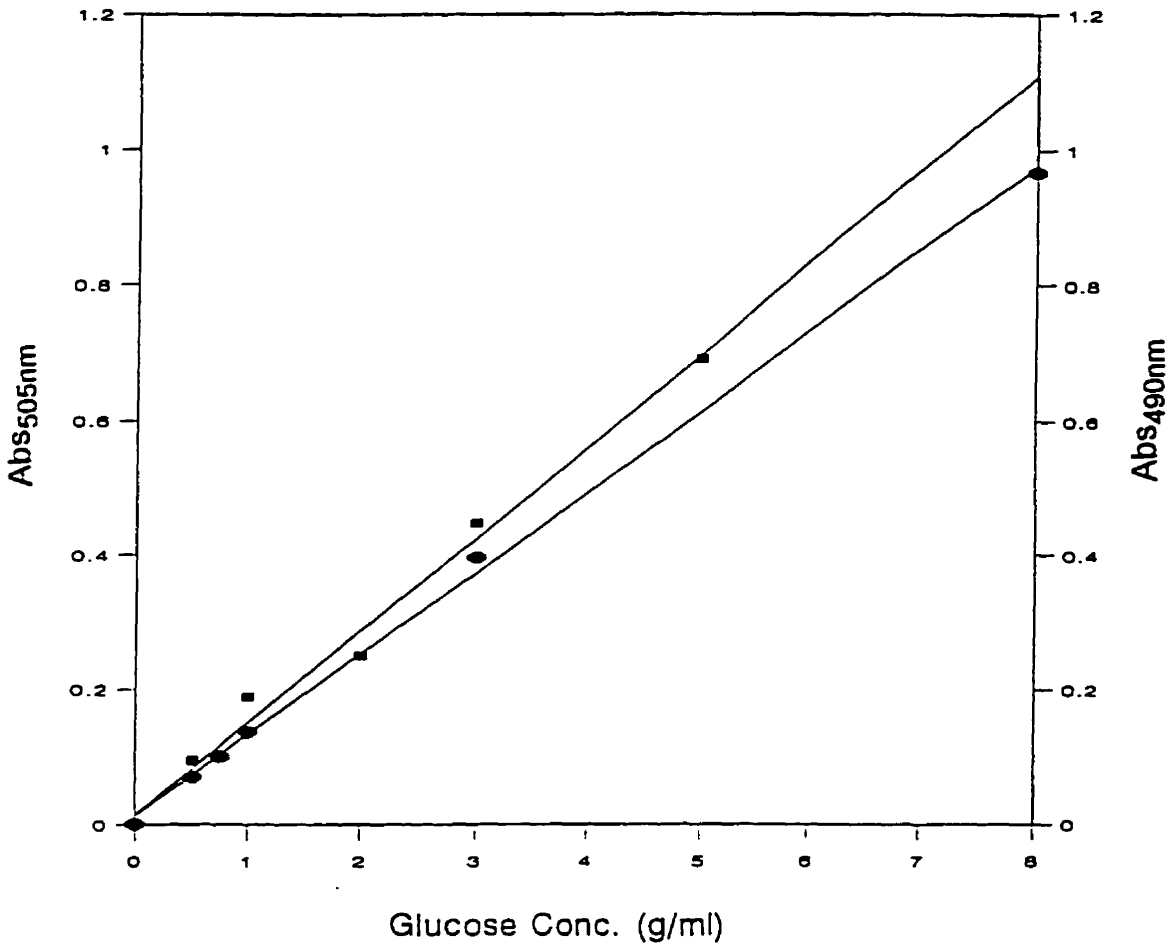


Figure 2.2. Calibration of glucose using the glucose (Trinder) test reagent on a Beckman spectrophotometer (●) and a Microplate reader (■) Abs_{505} (●): R^2 = 0.9999, Y = 0.0107 + 0.1194X, Abs_{490} (■): R^2 = 0.9884 Y = 0.0139 + 0.1368X.

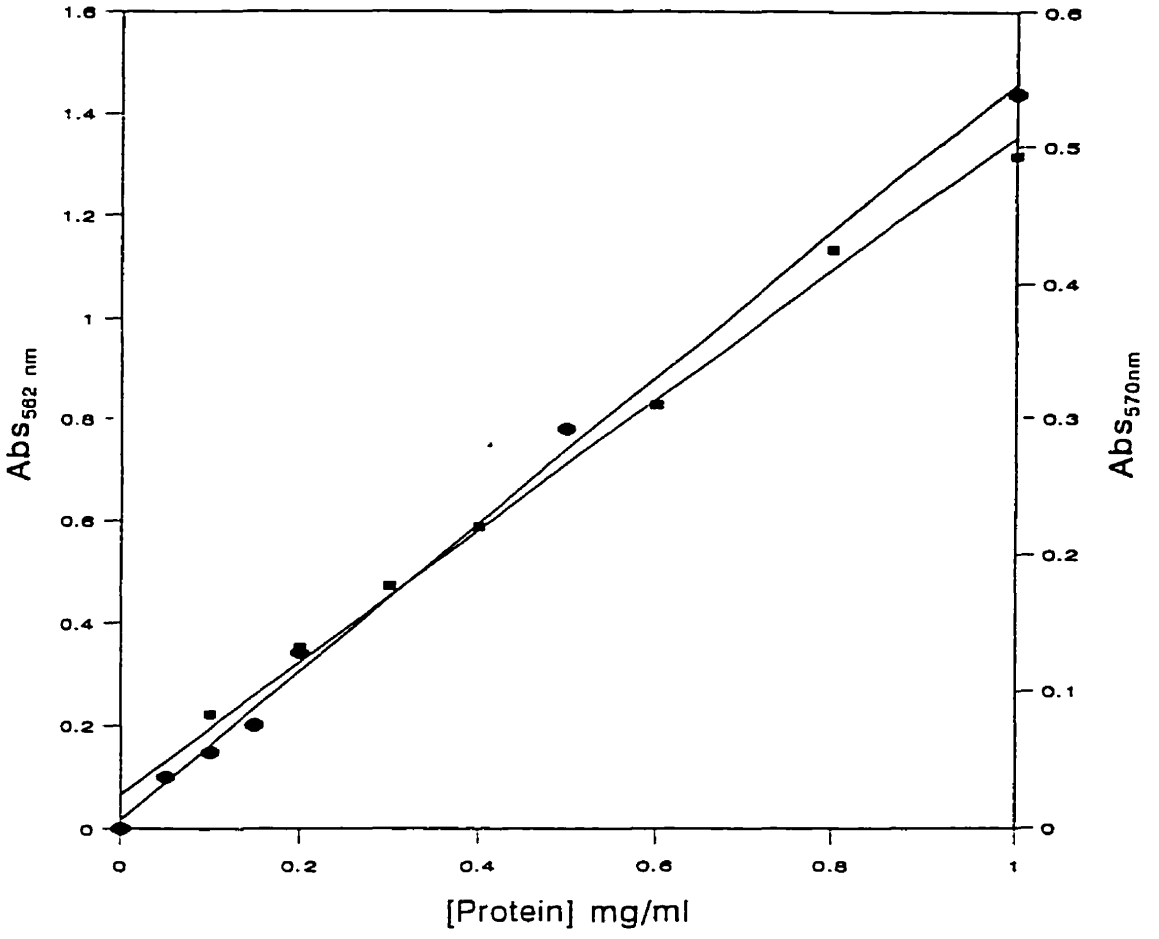


Figure 2.3. Calibration curve of bovine serum albumin using BCA protein assay reagent on a Beckman spectrophotometer (●) and a Microplate Reader (■). Abs_{562} (●.): R^2 = 0.9967, Y = 0.0177 + 1.4398X, Abs_{570} (■): R^2 = 0.9979, Y = 0.0299 + 0.0005.

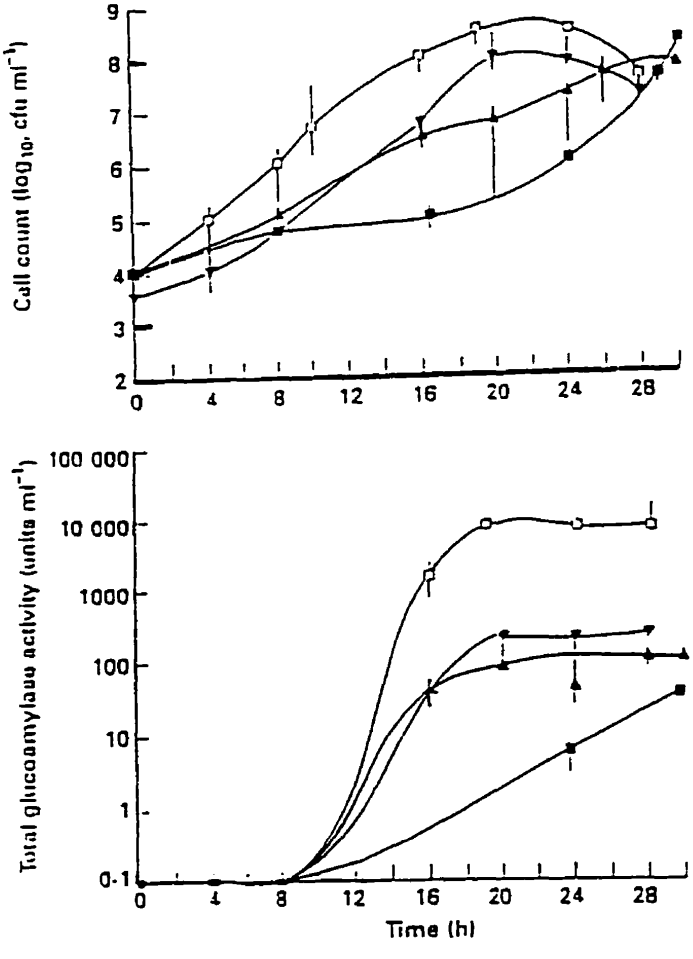


Figure 2.4. Effect of dextrin concentration on growth and glucoamylase activity of *Lactobacillus amylovorus* at 30°C with uncontrolled pH: (■), 0.1% (w/v) dextrin; (▲), 0.5% (w/v) dextrin; (□), 1.0% (w/v) dextrin; (▼), 1.5% (w/v) dextrin. The amount of enzyme represents intracellular enzyme activity.

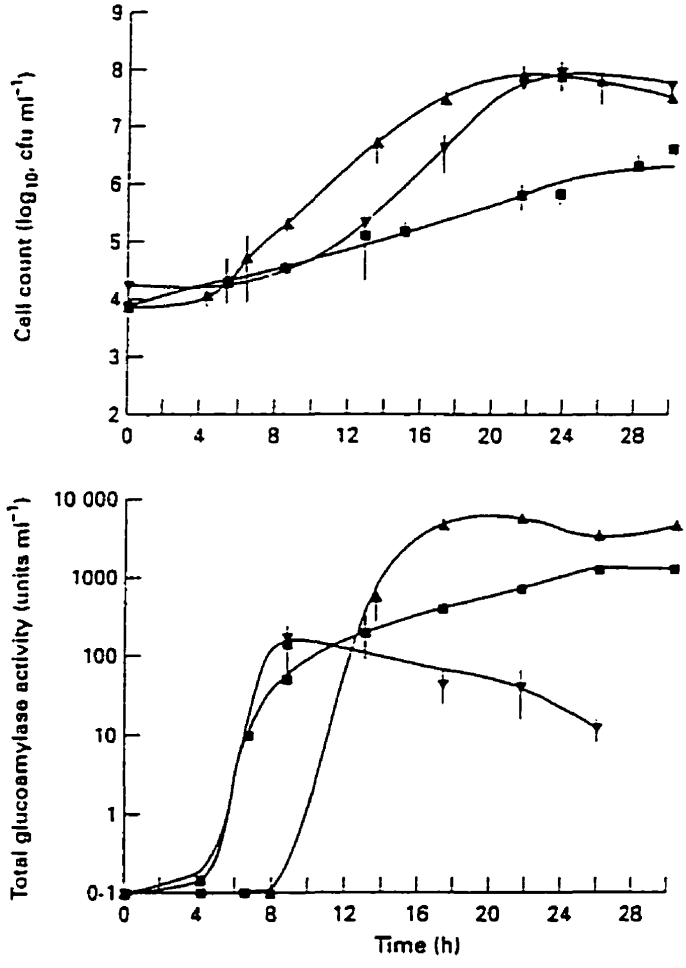


Figure 2.5. Effect of pH on growth and glucoamylase production of *Lactobacillus amylovorus* at 30°C, using 1.0% (w/v) dextrin (■, pH 4.5; ▲, pH 5.5; ▼, pH 6.5).

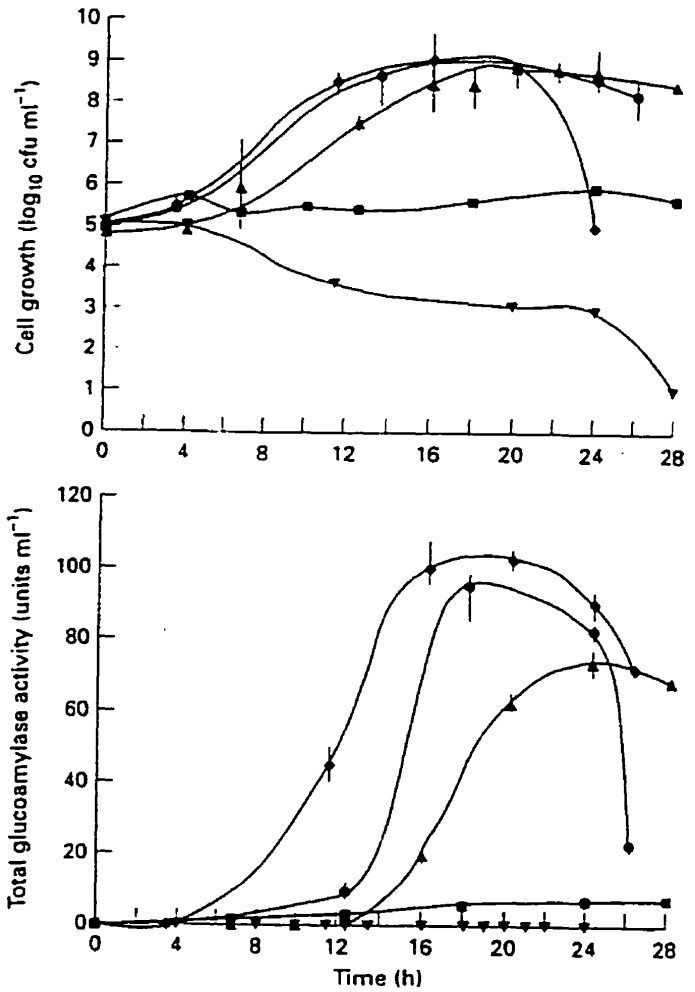


Figure 2.6. The effect of temperature on growth and glucoamylase production of *Lactobacillus amylovorus* at pH 5.5, using 1.0% (w/v) dextrin (■, 25°C; ▲ 30°C; ●, 37°C; ♦, 45°C; ▼, 55°C).

CHAPTER 3

PURIFICATION OF GLUCOAMYLASE FROM *LACTOBACILLUS AMYLOVORUS* ATCC 33621.

This chapter continues from previous work where the maximum conditions for growth and glucoamylase production were established. These conditions were then used to produce cell mass from which protein was extracted and glucoamylase was then purified. The molecular mass, pI, amino acid composition, N-terminal sequence, and other properties of the purified enzyme were identified.

This chapter was summarized and the key results were submitted as a manuscript for publication in the journal, "Current Microbiology". The manuscript entitled "Purification of glucoamylase from *Lactobacillus amylovorus*" was co-authored by Jennylynd A. James, Jean-Luc Berger and Byong H. Lee. The methodology used for protein purification was a collaboration of ideas of all the co-authors. This work was supervised by Jean-Luc Berger and Byong Lee. The actual experimental work, and writing of the manuscript were done by Jennylynd James. Co-authors Jean-Luc Berger and Byong Lee edited the manuscript prior to submitting it for publication. The N-terminal amino acid sequence and amino acid composition of the purified protein were determined at Queen's University and the University of Toronto, respectively.

3.1 ABSTRACT

An intracellular glucoamylase (E.C. 3.2.1.3) was purified to homogeneity from *Lactobacillus amylovorus* on a Fast Protein Liquid Chromatography System (FPLC) using a Mono Q ion-exchanger and two Superose 12 gel filtration columns arranged in series. The enzyme activity was quantified using a specific, chromogenic substrate, *p*-nitrophenyl-ß-maltoside. Preparative gel electrophoresis was then used to further purify active enzyme fractions. Native polyacrylamide gel electrophoresis (Native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme showed a single protein band of molecular weight 47 kDa. Glucoamylase activity of the purified protein was confirmed by its ability to degrade starch on a 0.025% starch-polyacrylamide gel stained with I_2/KI. Glucoamylase exhibited optimum catalytic activity at pH 6.0 and 45°C and the enzyme had an isoelectric point near 4.39. The glucoamylase contained high levels of hydrophilic amino acids which is comparable to fungal glucoamylases.

3.2 INTRODUCTION

Glucoamylase (EC 3.2.1.3) is an exo-acting carbohydrase which liberates ß-D-glucose from the non-reducing chain-ends of oligosaccharides by hydrolysing α-1,4 linkages consecutively. The primary application of glucoamylases

commercially is the production of glucose syrups from starch. These syrups can be used for fermentation, production of crystalline glucose, or as a starting material for fructose syrups (Fogarty, 1983). Glucoamylases have also been applied to the brewing industry in the production of low calorie beer where maltodextrins in malted barley are hydrolysed to simple sugars which can then be completely fermented by brewers yeast. The typical enzyme of choice in the food industry is the thermostable enzyme obtained from fungi, since this permits rapid reactions at high temperatures. Molds which have been used for commercial glucoamylase production include *Aspergillus* sp. (Ghosh *et al.* 1990; Pandey and Radhakrishnan, 1993) and *Rhizopus* strains (Ohnishi *et al.* 1990; Yu and Hang, 1991).

Several anaerobic bacteria have been investigated for their ability to produce starch-hydrolysing enzymes (Hyun and Zeikus, 1985; Ohnishi *et al.* 1991; Specka *et al.* 1991; Chaga *et al.* 1993). The glucoamylases from these strains have all been thermostable thus demonstrating possible applications in the saccharification of starch to glucose. In low calorie beer production, there is a need for a thermolabile glucoamylase which can be more readily inactivated by mild pasteurization (60-65°C, 6 min/ 71-74°C, 15 sec) used in the final stage of beer manufacture. This would solve the problem of change of flavor and product sweetening during storage.

Some microbial source of such a glucoamylase, or the cloning of the microbial gene coding for glucoamylase into Brewer's yeast (*Saccharomyces cerevisiae*) would be of great benefit, eliminating the addition of commercially

prepared enzymes and reducing overall production costs. This paper describes the purification of glucoamylase from a "food grade" bacteria, *Lactobacillus amylovorus*. This organism was first isolated from cattle waste-corn fermentations (Nakamura, 1981) and is known to secrete large quantities of α-amylase (Burgess-Cassler and Imam, 1991). *Lb. amylovorus* has also been found to produce other amylolytic enzymes which have not yet been identified or characterized (Fitzsimons and O'Connell, 1994). From preliminary experiments, glucoamylase production from *Lb. amylovorus* was found to be intracellular with maximal growth and glucoamylase production occurring in MRS medium containing 1% dextrin, at pH of 5.5 and 37°C-45°C (James and Lee, 1995).

We undertook the present study to purify glucoamylase from *Lactobacillus amylovorus* and estimate apparent native and subunit molecular weight, isoelectric point, as well as amino acid composition of the glucoamylase active fraction. We used a "novel" glucoamylase assay which employs the specific substrate *p*-nitrophenyl ß-maltoside (McCleary *et al.* 1991). This substrate is not hydrolysed by α-amylase, α-glucosidase and transglucosidase which appear as contaminants in commercial glucoamylase preparations (McCleary *et al.* 1991). The optimal pH and temperature of glucoamylase from *Lb. amylovorus* are desirable for application in the brewing of low calorie beer. Investigation of the properties of glucoamylase from *Lb. amylovorus* will assist further in determining the suitability of the enzyme for food industry applications.

3.3 MATERIALS AND METHODS

3.3.1 Organism and crude extract preparation

Lactobacillus amylovorus ATCC 33621 obtained from American Type Culture Collection (Maryland) was activated by two successive transfers in MRS (de Man *et al.* 1960) broth (Difco Laboratories, Detroit, MI) and maintained in a sterile broth/glycerol (60%:40%, v/v) solution at -40°C. Working cultures were prepared by two successive transfers of stock culture to MRS broth at 18 h intervals at 37°C. Cell culture (0.1% v/v) was inoculated in MRS medium without dextrose (Institut Rosell Inc., Montreal, Québec, Canada), with the addition of 1.0% potato dextrin (Sigma Chemicals, St. Louis, MO). Growth was carried out in a laboratory fermenter (1.5 L) at 37°C, 70 rpm, with the pH adjusted to 5.5. The preparation of crude cell-free extracts was performed as previously described (James and Lee, 1995). Nucleic acids in crude extract were digested by the addition of DNAse (2.5 µg/ml, final concentration) (Pharmacia, Baie d'Urfé, Québec, Canada) and RNAse (5.0 µg/ml, final concentration) (Boehringer Mannheim, Laval, Québec, Canada), leaving the mixture overnight at 4°C. Heavy metals were precipitated by addition of ethylenediaminetetraacetic acid (EDTA) (Sigma) to a final concentration of 0.01 M, without the loss of glucoamylase activity.

3.3.2 Chemicals, reagents and equipment

All reagents were purchased from Sigma unless otherwise specified. The substrate *p*-nitrophenyl-ß-maltoside (Megazyme (Australia) Pty. Ltd., N.S.W., Australia) was used to assay glucoamylase activity. All other chemicals were of analytical reagent grade. A Fast Protein Liquid Chromatography (FPLC) system (Pharmacia) was used for purification of the enzyme. It consisted of two P-500 pumps, two injection valves, MV-7, with the appropriate loops and superloops for sample injection, an LCC-501 Plus controller, UV monitor, UV M II (having 1-cm optical path) set at 280 nm, a fraction collector, FRAC-100, and a REC-102 dual pen recorder. Prepacked chromatography columns of Mono Q HR 16/10 (preparative, 16 cm x 1 cm i.d.) and Superose 12 HR 10/30 (30 cm x 1 cm i.d.) from Pharmacia were used for enzyme purification. The system was programmed to operate automatically. Preparative gel electrophoresis was performed using the Mini Protean II and Large Slabgel systems from Biorad (Mississauga, ON, Canada). The gel nebulizer, micropure separator and microconcentrator supplied by Amicon (Oakville, Ontario, Canada). Gel electrophoresis was also performed using the Phast system (Pharmacia) for miniaturised gels: native (8-25%), SDS (12.5%), and isoelectric focusing (pH 4-6). Gels, staining dye, and molecular mass markers were supplied by Pharmacia and BioRad and membranes for concentrating protein solutions were supplied by Amicon. Calibration proteins for gel chromatography were supplied by Boehringer Mannheim.

3.3.3 Enzyme Assays

Glucoamylase activity was determined spectrophotometrically by a modification of the method of McCleary *et al.* (1991). A 100 µl portion of enzyme solution was added to 100 µl of *p*-nitrophenyl-ß-maltoside solution (Megazyme). The mixture was incubated at 40°C for 10 min and terminated with color developed by the addition of 1.5 ml of 2% Trizma base (Sigma). Activity was calculated as follows: Activity (Units/ml) = $\blacktriangle A_{410}/t$ x a/b x 1/17.8 x Dilution Factor, where $\blacktriangle A_{410}$ = Absorbance (reaction) - Absorbance (blank), t = Incubation time, a = Final reaction volume, b = Volume of enzyme and 17.8 = ε_mM *p*-nitrophenol in 2% Trizma base.

One unit of enzyme activity is defined as the amount of enzyme required to release one µmole of *p*-nitrophenol from the substrate per min under defined assay conditions.

In cases where analyses required a change in pH or temperature an alternative assay using an oligosaccharide substrate was used. Glucoamylase activity was determined by a modification of the method of Ghosh *et al.* (1990). A 100 µl aliquot of cell extract was incubated with 100 µl of 1% (w/v) soluble starch (Sigma) in 0.05 M sodium phosphate buffer (pH 6.0) at 45°C for 30 min. The reaction was terminated by immersing the tube in a boiling water bath for 5 min. The glucose liberated by enzymatic action was measured by a glucose oxidase method (Glucose-Trinder test kit, Sigma), using glucose standards (Sigma) for calibration. One unit of glucoamylase was defined as the amount of enzyme

required to release 1 µmole of glucose per min under assay conditions. Specific activity was defined as enzyme units per mg protein.

3.3.4 Protein assay

Protein was determined spectrophotometrically by the Bicinchoninic Acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as a standard (Smith *et al.* 1985).

Protein concentrations in the column eluates were estimated from the absorbance at 280 nm.

3.3.5 Protein purification

3.3.5.1 Ion Exchange Chromatography

A preparative anion exchange column (Mono-Q HR 16/10) was equilibrated with 0.02 M Triethanolamine/HCl buffer (pH 7.5) (TEA/HCl), 0.01 M EDTA. About 100 mg of crude extract was loaded onto the column at a flow rate of 2 ml/min. After washing the column with the equilibration buffer, the enzyme was eluted using a linear gradient of sodium acetate (0.1 - 0.4 M) in the same buffer. Fractions (6 ml) were collected and tested for glucoamylase activity. Those fractions with the highest activity were pooled and concentrated by ultrafiltration using a Centriprep-10 concentrator (exclusion limit 10 kDa, Amicon).

3.3.5.2 Gel Filtration Chromatography

The active enzyme fractions were applied to two Superose 12 HR 10/30 gel filtration columns arranged in series. Proteins were eluted in 0.05 M TEA/HCl (pH 4.5) at a flow rate of 0.15 ml/min and active fractions were pooled and concentrated. Fractions (3 ml) from each protein peak were collected and tested for glucoamylase activity. Active fractions were pooled and concentrated using the Centricon-10 concentrator. Protein was desalted for further purification using a PD-10 size exclusion column (Pharmacia) with elution in 0.01 M TEA/HCL pH 7.0.

3.3.5.3 Preparative Gel Electrophoresis

A 7.5% polyacrylamide native slabgel was prepared according to the method supplied by BIORAD, using the Protean™ large slab gel apparatus and approximately 0.5 mg of protein was loaded. One portion of the gel was stained for proteins using a gel staining solution (0.1% Coomassie blue R250 in fixative, 40% methanol, 10% acetic acid). The gel was destained with 40% methanol/10% acetic acid to remove background. The other portion of the gel was soaked in 0.05 M sodium acetate buffer (pH 4.5) for 10 min to exchange buffers, then the slab gel was cut into lateral measured strips which were each cut into very fine pieces to allow for a large surface area for elution. Protein elution from gel was further enhanced using a Gel Nebulizer™ (Amicon) according to the manufacturer's instructions. The Gel Nebulizer is a device for the conversion of a gel slice into a

fine spray, driven by centrifugal force. Extruded buffer containing protein passed through the Micropure separator (0.22 µm) and was retained by the ultrafiltration membrane in the Microcon concentrator (10 kDa cut-off) for simultaneous concentration and/or desalting (Krowczynska *et al.* 1995). Glucoamylase activity in eluted proteins was detected by reaction with *p*-nitrophenyl-ß-maltoside. By comparing the protein stained bands with the glucoamylase active strips, the protein with glucoamylase activity was identified.

3.3.5.4 Enzyme purity determination

The purity of the enzyme at each step in purification was examined by Native-PAGE and SDS-PAGE using the method of Laemmli (1970). Mini-gels (5 cm x 4 cm) were run on a Phast electrophoresis system (Pharmacia) for 45 min at 15°C and 30 V/cm. A 4% stacking gel and 8-25% polyacrylamide gradient was used for Native-PAGE and a 12.5% homogeneous gel was used for SDS-PAGE. The protein samples were mixed 1:1 (v/v) with sample buffer (20 mM Tris/HCl, 2 mM EDTA, 5% SDS, 10% ß-mercaptoethanol, pH 8.0), and boiled for 5 min before applying to the SDS-polyacrylamide gel. Fast Coomassie staining was done with 0.1% PhastGel Blue R (Pharmacia) in 10% methanol-30% acetic acid-water (1:3:6, v/v/v). The gel was destained in an aqueous mixture containing 10% acetic acid and 30% methanol. Gels were preserved in 5% glycerol and 10% acetic acid.

3.3.5.5 Apparent molecular mass determination

The apparent molecular mass of the purified enzyme was determined by SDS-PAGE using a 12.5% polyacrylamide gel with the "Phast System" (Pharmacia). The following low molecular weight proteins were used as standards: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

Molecular mass was also estimated by gel-filtration on two Superose 12 column (30 cm x 1.0 cm i.d.) arranged in series. The columns were pre-equilibrated with 0.05 M TEA/HCl buffer, (pH 4.5) at a flow rate of 0.15 ml/min. Calibration proteins for gel chromatography (Boehringer) were cytochrome c (12.5 kDa), chymotrypsinogen A (25 kDa), albumin (hen) (45 kDa), bovine serum albumin (68 kDa), aldolase (158 kDa) and catalase (240 kDa). A calibration curve was constructed by plotting the partition coefficient (K_{av}) versus molecular mass, where $K_{av} = (V_e-V_o)/(V_t-V_o)$ with V_e = Elution volume of Protein, V_t = Total column volume and V_o = Void volume.

3.3.6 Properties of the purified enzyme

3.3.6.1 Effects of pH and temperature

The effect of pH on the activity of purified enzyme was measured in 0.05

M citrate phosphate buffer (pH 3.0 to 5.0), 0.05 M sodium phosphate buffer (pH 6.0 to 8.0). Enzyme solution (100 µl) was incubated with 100 µl of 1% (w/v) starch solution in the appropriate pH buffer, for 30 min at 45°C. The glucose released was measured using the glucose oxidase method (Sigma). The experiment was conducted in triplicate.

The effect of temperature on glucoamylase activity was measured in the range of 21°C-80°C. A solution of 1% (w/v) starch in 0.05 M sodium phosphate buffer (pH 6.0) was equilibrated for 2 min at the test temperature before an equal volume of crude enzyme was added. The mixture was then incubated for 30 min at the test temperature and enzyme activity assayed by measuring the amount of glucose liberated.

3.3.6.2 Isoelectric focusing

The Phast system was used to carry out isoelectricfocusing of the purified enzyme according to the instructions provided by Pharmacia. A PhastGel IEF medium, pH range of 4.0 - 6.5 (homogeneous polyacrylamide, 5% T, 3% C) was used. The gel was run at 2000 V, 5.0 mA, 3.5 W and 15°C for 25-30 min. Pharmalytes in the pH range of 4.0 - 6.5 were used as carrier ampholytes, which form stable pH gradient across the gel between two electrodes. Electrophoresis was carried out according to the manufacturer's instructions. Low isoelectric point (pI) calibration proteins (Pharmacia) were used to estimate the isoelectric point of

the pure protein: pepsinogen (pI 2.8), amyloglucosidase (pI 3.5), methyl red (dye, pI 3.75), glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), ß-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55). A calibration curve was constructed using the migration distance from the cathode versus the pI for each protein and the isoelectric point was estimated from this curve.

3.3.6.3 Activity Staining

The enzyme activity was confirmed by carrying out Native-PAGE in a 3.7% polyacrylamide gel containing 0.45% starch as outlined in the method by Fitzsimons and O'Connell (1994). Portions (15 µl) of enzyme solution (0.92 mg/ml protein) containing 5 µl of 20% (w/v) glycerol and 10% (w/v) bromophenol blue were applied to stacking gel wells. Each sample was replicated on the gel so that after electrophoresis half of the gel could be used for protein staining and the other half for activity staining.

Starch degrading activity was visualized by using modifications of the methods by Kakefuda and Duke (1984). The gel was equilibrated in 0.05 M sodium citrate buffer at pH 6.0 for 5 min, followed by immersion in 2% (w/v) starch (dissolved in 0.05 M sodium citrate buffer pH 6.0) for 20 min at room temperature. The gel was then incubated for 10 min at 45°C in the same buffer without starch. Excess buffer was drained off and the gel was stained with KI/I reagent (1 mM I_2,

0.5 M KI). The gel was incubated at room temperature to allow the blue background color to develop, then excess KI/I reagent was removed by rinsing with distilled water. The gel was then refrigerated to promote starch precipitation, and later photographed.

3.3.6.4 Amino acid analysis and N-terminal sequence

Samples for amino acid analysis and N-terminal sequencing were prepared by a process of transferblotting to Polyvinylidene fluoride (PVDF) membrane under reducing conditions according to the method of BioRad. A 7.5% SDS polyacrylamide gel was prepared and SDS-PAGE was conducted using the purified protein and low molecular mass protein standards. The Mini Trans-Blot Electrophoretic transfer Cell (Biorad) was then used for blotting at 100 V for 1 h, with the temperature maintained below 30°C, with a cooling unit. The membrane was stained with 0.2% (w/v) coomassie brilliant blue R-250 in 45% methanol and 10% (v/v) acetic acid, for 2 min. The PVDF membrane was then destained with a solution of 45% methanol and 7% acetic acid. When the bands were clearly visible, the membrane was washed well with distilled, deionized water and air dried for analyses.

The amino acid composition of the purified enzyme was determined at the HSC Biotechnology Service Centre, University of Toronto (Ontario, Canada). The dried protein was hydrolysed for 24 h in 6 M HCl with 1% phenol. The sample was

then treated with a redrying solution consisting of methanol:water:triethylamine (2:2:1) and dried under vacuum. The sample derivatized for 20 min at room temperature using a solution of methanol:water:triethylamine:phenylisothiocyanate (PITC) (7:1:1:1) was injected into the Waters PICO-TAG System (Waters, Toronto, ON, Canada), running at 33°C for detection of amino acids (Heinrickson and Meredith, 1984). Cysteine content was obtained from a performic acid oxidized sample.

N-terminal sequencing of the purified enzyme was conducted on a pulsed liquid sequencer model 473A equipped with a microgradient for phenylthiohydantoin (PTH) analysis (Queen's University, London, ON, Canada).

3.4 RESULTS

3.4.1 Purification of glucoamylase

Conditions for optimal protein separation and yield were obtained by conducting several chromatographic runs using the FPLC System. Purification of glucoamylase is summarized in Table 3.1. Crude extract was treated with DNAse and RNAse to reduce the nucleic acid content thus facilitating preparative ion-exchange chromatography. The elution profile of crude extract from *Lb. amylovorus* by ion-exchange chromatography (Mono-Q HR 16/10) is shown in Fig. 3.1. Glucoamylase activity is detected in fractions eluted within 0.22-0.29 M sodium

acetate. The elution profile of active enzyme fractions from gel filtration chromatography is shown in Fig. 3.2 and glucoamylase activity was eluted in the fraction collected at 18.5 ml. At this step, the enzyme was purified about 8 fold with a 21% yield. Preparative gel electrophoresis was performed to remove contaminating proteins and obtain a pure enzyme eluted from polyacrylamide gel for characterization.

3.4.2 Enzyme purity and Apparent Molecular Mass

Glucoamylase activity of the eluted protein was confirmed by testing enzyme activity of various portions of the gel. The glucoamylase active protein band was located at R_f 0.7 on a native slab gel (Fig. 3.3). Both Native and SDS-PAGE showed a single protein band for the purified enzyme, indicating that glucoamylase was a monomer (Figs. 3.4 & 3.5). Native-PAGE patterns of proteins at different stages of purification and the activity stain of the purified enzyme on a starch/polyacrylamide gel are shown in Fig. 3.4. The R_f value of the zone of clearing formed by a starch-degrading enzyme, matched the R_f value of the purified protein. The apparent molecular mass of glucoamylase as estimated from the calibration curves of molecular mass protein standards run on SDS-PAGE. Fig. 3.6 shows the proteins used to prepare the standard curve and Fig. 3.7 gives the linear regression of molecular mass versus the R_f value for each protein. Fig. 3.8 shows the calibration curve prepared using gel filtration chromatography. Molecular

mass estimates for the purified proteins were 47,179 Da and 57,650 Da for SDS-PAGE and gel filtration chromatography, respectively. Other researchers observed this difference in molecular mass when gels were used for determination of bacterial amylase, fungal amylase and α-glucosidase (Shantha Kumara *et al.* 1993). The differences are thought to be due to interactions between polysaccharide-splitting enzymes and the gels.

3.4.3 Optimal pH, temperature and pI

The optimum pH of glucoamylase activity was about 6.0 (Fig. 3.9). Temperature dependence of the enzyme was determined between 21-80°C and the optimum temperature for enzyme activity was 45°C (Fig. 3.10). The isoelectric point was found near pH 4.39 when estimated from the calibration curve (Fig. 3.11).

3.4.4 Amino acid composition and N-terminal sequence

The amino acid composition of purified glucoamylase from *Lactobacillus amylovorus* is compared with the amino acid composition of other glucoamylases in Table 3.2. Hydrophobic amino acids like Phe, Met, Tyr and Ile occurred in lower concentrations than the more acidic amino acids like Glu and Asp. The partial N-terminal sequence of the glucoamylase active protein was found to be: Ala-Lys-

Asp-Ile-Val-Asp-Asp-Leu-Asp-Val-Asn-Gly-Lys-Lys-Val-Ile-Met-Arg-Val-Asp (Table 3.3).

3.5 DISCUSSION

An optimal activity in the acid pH range would be beneficial in the brewing of beer, since during the fermentation of wort by *Saccharomyces cerevisiae*, the pH of wort lowers to about 4.0. The carbon dioxide produced dissolves in the wort to the extent of about 0.3% by weight (Potter, 1986). Continued maltodextrin degradation at low pH would provide more simple sugars for fermentation and thus ensure a lower calorific value of beer. Glucoamylases tend to have pH optima ranging from 3.5 to 6.5 (Vihinen and Mäntsälä, 1989) and the pH of glucoamylase from *Lb. amylovorus* was found to be in a useful range for application in the brewing industry. Beer in bottles and cans is pasteurized at about 60°C for 6 min within the container as a measure of prolonging shelf life. This is required after the enzyme has completed its function of saccharification. A temperature optimum of 45°C would predispose glucoamylase from *Lb. amylovorus* to heat inactivation by these temperatures and again provides a useful property in low calorie beer production.

Glucoamylase from *Lb. amylovorus* was found to be a relatively small molecule of mass 47 kDa. The molecular mass of other glucoamylases range between 26,859 Da and 112,000 Da and since these enzymes are found mainly

in molds, glycosylation of the enzyme would account for some of the high molecular weights (Fogarty, 1983). The isoelectric point pI of glucoamylase from *Lb. amylovorus* was 4.39 while isoelectric points of glucoamylases range from 3.2 in *Aspergillus niger* 152 to 9.1 in *Rhizopus niveus* (Vihinen and Mäntsälä, 1989).

The presence of α-amylase and other contaminants in *Lb. amylovorus* were thought to have a synergistic effect on glucoamylase activity in crude extract, since the yield of glucoamylase activity decreased significantly as the enzyme was separated from other intracellular proteins (Burgess-Cassler and Imam, 1991; Fitzsimons and O'Connell, 1994). Amino alcohols like Tris are known to inhibit glucoamylases (Shantha Kumara *et al.* 1993) and so the presence of Tris in the buffer system of Preparative PAGE plus the operating pH of 8.3 may have affected glucoamylase stability and activity. This resulted in low yields of the final purified protein. However, glucoamylases employed in the food industry are used in a partially purified form, the main focus being on the specific activity of the enzyme.

The high cost of purifying an intracellular protein precludes this enzyme from competing with the inexpensive extracellular glucoamylases produced from mold strains commercially. But, the low optimal temperature of glucoamylase from *Lb. amylovorus* would be a benefit in the brewing of low calorie beer and this property may be incorporated in the genetic engineering of a brewer's yeast strain expressing the gene coding for this enzyme.

Glucoamylase from *Lb. amylovorus* contained low levels of Thr but high levels of Ser. It is not known if this high level of Ser enhances enzyme activity

since bacterial enzymes tend not to be glycosylated. Glucoamylases from mold strains have been reported with low hydrophobic amino acid contents (Fogarty, 1983). They however show very high levels of Ser and Thr which have -OH- side chains used for the attachment of glycolytic groups, since all mold glucoamylases are glycoproteins. The amino acid composition of glucoamylase from *Halobacterium sodomense* (Chaga *et al.* 1993) when compared with that of glucoamylase from *Lb. amylovorus* again showed low levels of hydrophobic amino acids. There is thus some correlation between amino acid composition and glucoamylase activity. The N-terminal amino acid sequence of glucoamylase active protein from *Lb. amylovorus* showed 35% homology with certain internal sequences of glucoamylase from *Clostridium* sp. when analyzed using the DNASIS database (Hitachi Software Engineering America, 1995) (Table 3.3). However, the data was not sufficient to be conclusive.

3.6 CONCLUSION

Glucoamylase from *Lb. amylovorus* has the advantage of being sensitive to ordinary pasteurization temperatures (60°-65°C for 6-8 min) which are adequate to inactivate the enzyme. In the brewing of low calorie beer this property would reduce the risk of residual enzyme activity remaining in the finished product, causing sweetening and off-flavors. The expensive process of isolating an intracellular enzyme does not make it competitive with commercial glucoamylases

from mold strains which are used at present. The properties of this enzyme should be studied further to determine its suitability for the brewing process. Moreover, overexpression or cloning of the gene coding for glucoamylase in brewer's yeast would be the best way to harness the advantageous properties of this glucoamylase while reducing the overall costs of brewing low calorie beer.

Table 3.1. Summary of the purification steps of glucoamylase from *Lactobacillus amylovorus* ATCC 33621.

Purification Step	Total protein (mg)	Total[a] activity (units)	Specific activity[b] (units/mg)	Yield (%)	Fold
1. Crude Extract	1358.03	9.10	0.0067	100	1
2. Ion-Exchange (preparative)	424.36	3.64	0.0086	40	1.3
3. Gel Filtration	35.94	1.93	0.0536	21	8
4. Preparative Gel[c] Electrophoresis	1.75	0.01	0.0057	0.13	-

[a]One unit of enzyme is defined as the amount of enzyme which releases one µmole of p-nitrophenol from the substrate per minute under defined assay conditions.
[b]Specific activity is defined as enzyme units per mg of protein.
[c]Purification data of preparative gel electrophoresis is approximate.

Table 3.2. Amino acid composition of glucoamylase from *Lactobacillus amylovorus* compared with other glucoamylases.

Amino acids	*Lb. amylovorus* (Res/1000)	*H. sodomense*[a]	*A. niger*[b]		*A. saitoi*[c]
			I	II	
Cysteine A	9	6	8	8	10
Aspartic acid	118	217	83	85	77
Glutamic acid	69	236	56	54	53
Serine	74	100	119	121	94
Glycine	137	161	59	63	61
Histidine	16	23	6	6	5
Arginine	29	59	23	22	18
Threonine	36	111	104	103	79
Alanine	105	145	78	82	70
Proline	38	103	29	29	29
Tyrosine	17	70	30	29	27
Valine	70	110	46	46	36
Methionine	19	12	4	4	2
Isoleucine	66	56	27	25	17
Leucine	78	114	53	56	46
Phenylalanine	33	77	27	28	21
Lysine	86	22	16	16	13
Tryptophan[d]	n.d.	37	30	32	28

[a] *Halobacterium sodomense* (Chaga *et al.* 1993)
[b] *Aspergillus niger* (Pazur *et al.* 1971), estimated from values for g/100g protein
[c] *Aspergillus saitoi* (Takahashi *et al.* 1981)
[d] Tryptophan was destroyed by acid hydrolysis
Units = Residues/1000 amino acids

Table 3.3. The N-terminal amino acid sequence of glucoamylase from *Lactobacillus amylovorus*, compared with the internal amino acid sequence of glucoamylase from *Clostridium* sp.

Organism	Sequences	Match (%)
	10 20	
Lb. amylovorus	AKDIVDDLDVNGKKVIMRVD	35
	• X: . . : : : ∧ v : . . . . . :	
Clostridium sp.	VNDIMTDLD -ENKQMTKDYD	
	880 890	

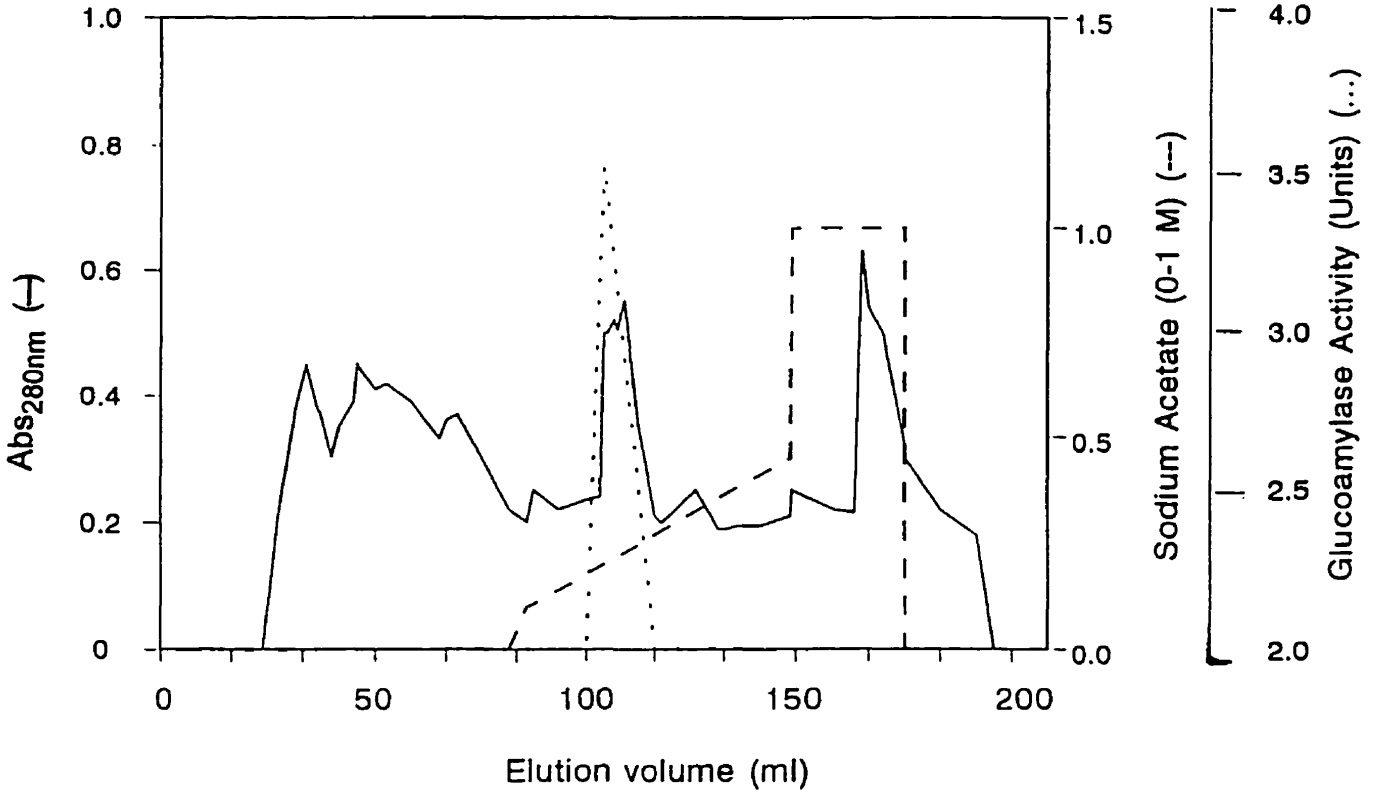


Figure 3.1. First ion-exchange elution profile of crude extract from *Lactobacillus amylovorus* ATCC 33621 on an ion exchange column (Mono Q HR 16/10). Enzyme activity (···), Protein at Abs_{280nm} (—), Salt gradient(---).

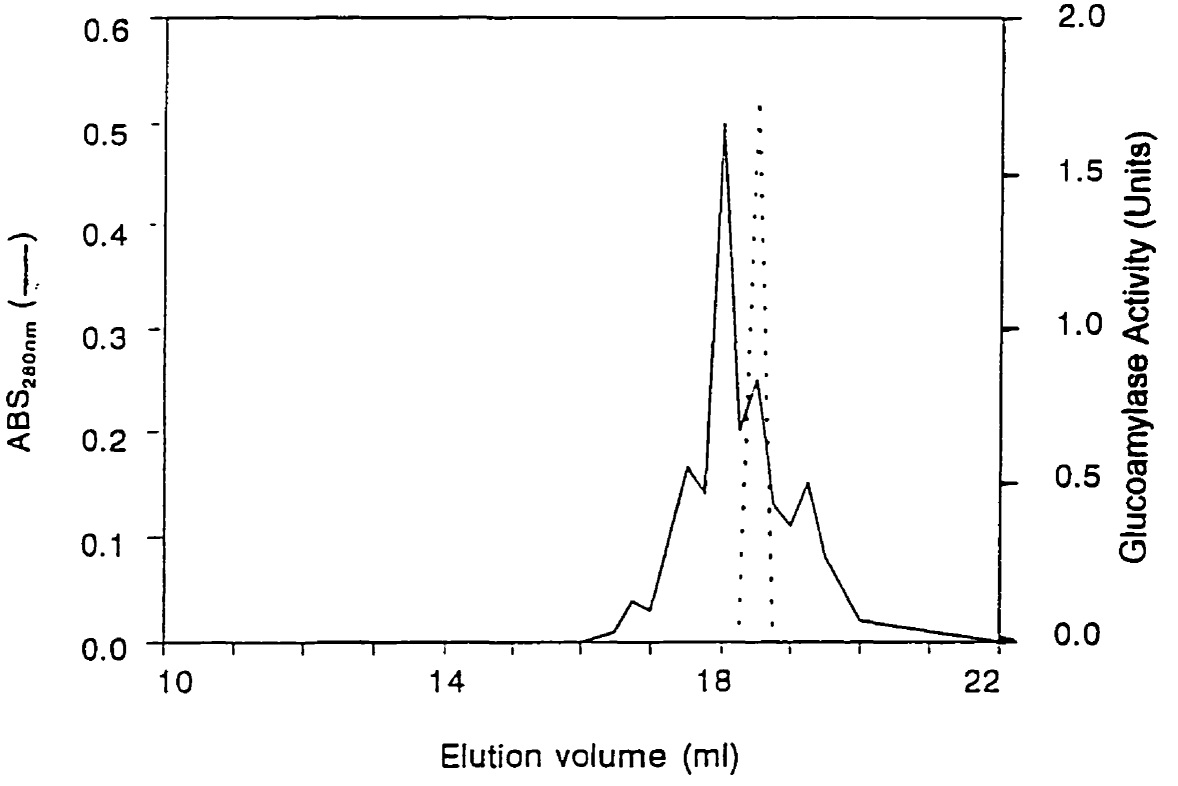


Figure 3.2. Elution profile of glucoamylase from *Lactobacillus amylovorus* by gel filtration chromatography (Superose 12 HR 10/30). Enzyme Activity (···), Protein at Abs_{280nm} (—).

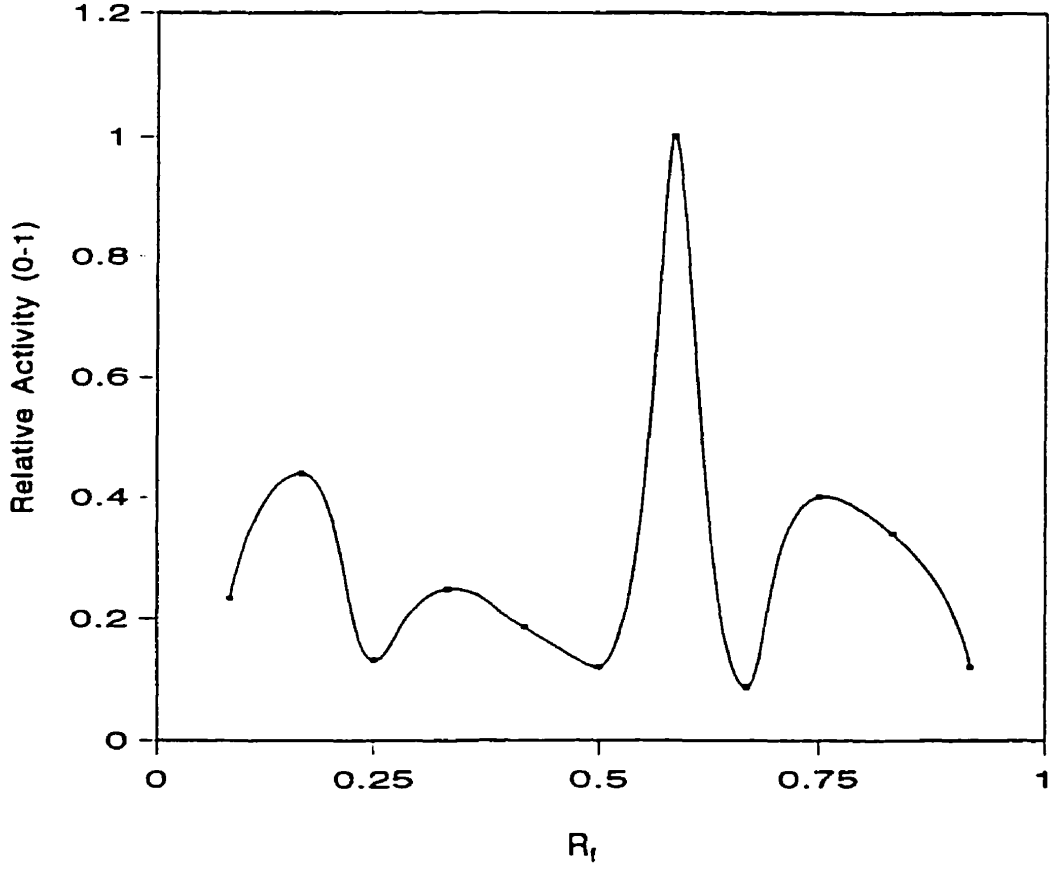


Figure 3.3. Preparative slab gel electrophoresis using glucoamylase active fractions obtained from gel filtration on 7.5% acrylamide gel. The protein was run on Native PAGE and glucoamylase activity versus R_f of gel strips were plotted.

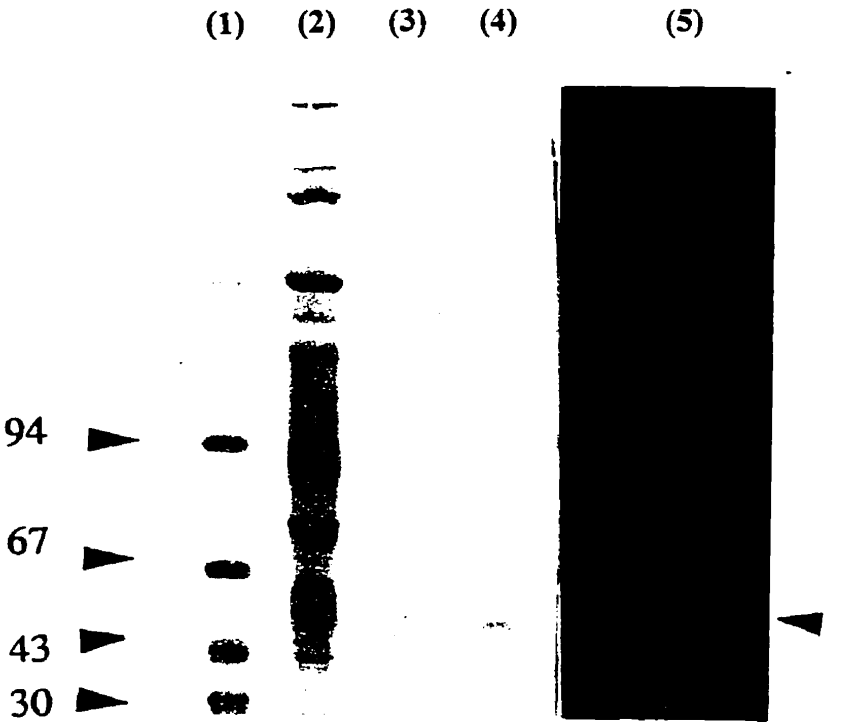


Figure 3.4. Native polyacrylamide gel electrophoresis (Native-PAGE) patterns of *Lactobacillus amylovorus* glucoamylase fractions obtained from different purification steps, on 8-25% gradient acrylamide gel (Coomassie blue stain). Lanes: 1, marker proteins; 2, after first ion-exchange chromatography; 3, after gel filtration; 4, after preparative gel electrophoresis; 5, activity staining of purified enzyme. Molecular mass markers in kilodaltons (top to bottom): Phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20.1), and α-lactalbumin (14.4).

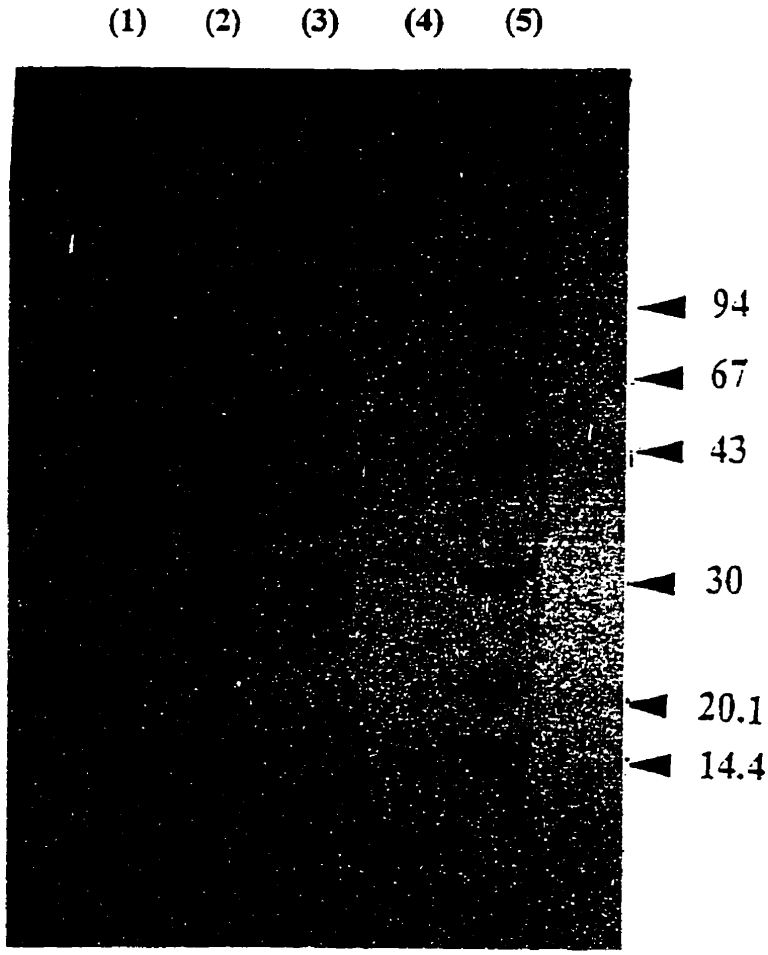


Figure 3.5. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) patterns of *Lactobacillus amylovorus* glucoamylase fractions obtained from different purification steps, on 12.5% gradient acrylamide gel (Coomassie blue stain). Lanes: 1, crude extract; 2, after first ion-exchange chromatography; 3, after gel filtration; 4, after preparative gel electrophoresis; 5, marker proteins. Molecular mass markers in kilodaltons (top to bottom): Phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20.1), and α-lactalbumin (14.4).

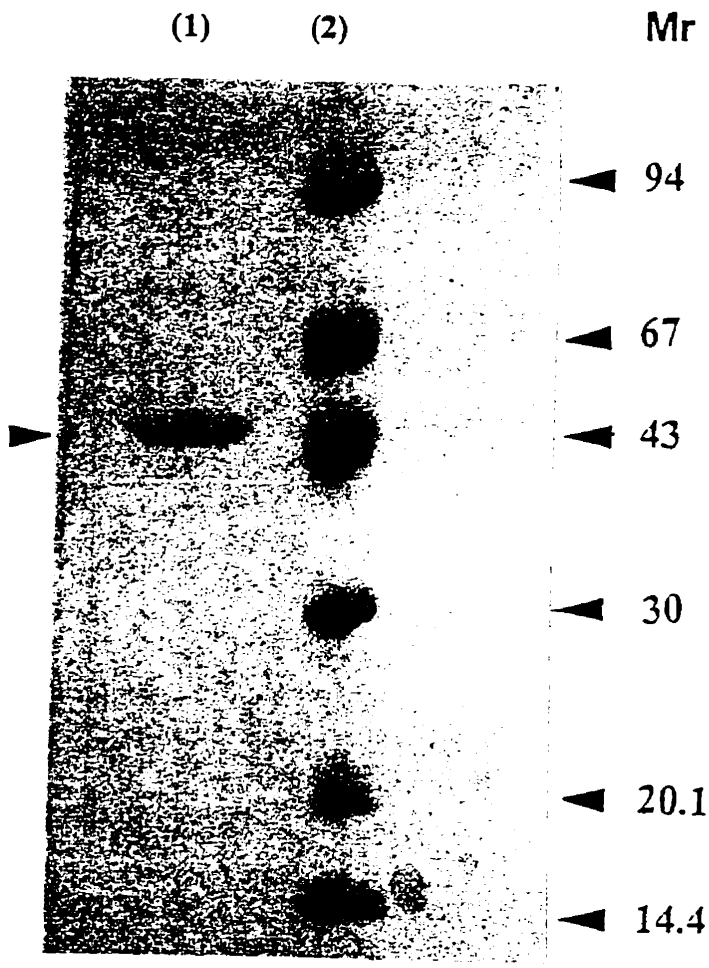


Figure 3.6. SDS-PAGE of purified glucoamylase from *Lactobacillus amylovorus* ATCC 33621 on 12.5% acrylamide gel. Lanes: 1, purified glucoamylase; 2, marker proteins, (Mr). Molecular mass markers in kilodaltons (top to bottom): Phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20.1), and α-lactalbumin (14.4).

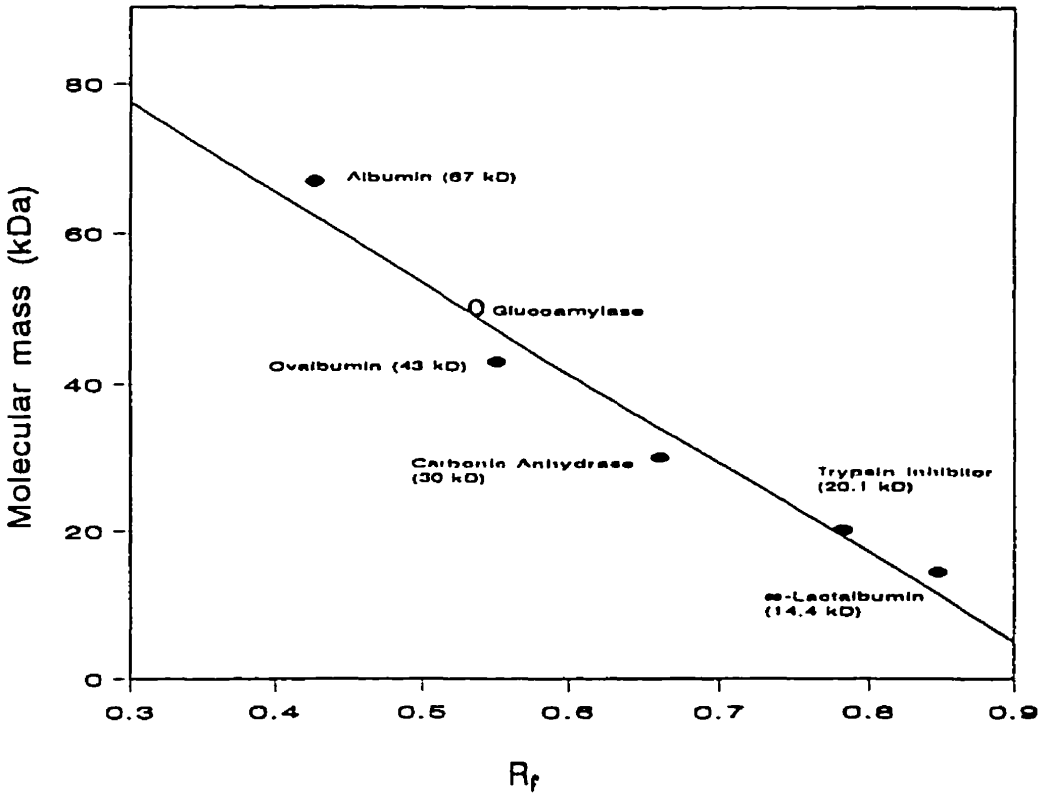


Figure 3.7. Estimation of molecular mass of glucoamylase from *Lactobacillus amylovorus* by SDS-PAGE (12.5% polyacrylamide) using low molecular mass markers. R^2=0.9824, Y = 113.41 - 120.34 X.

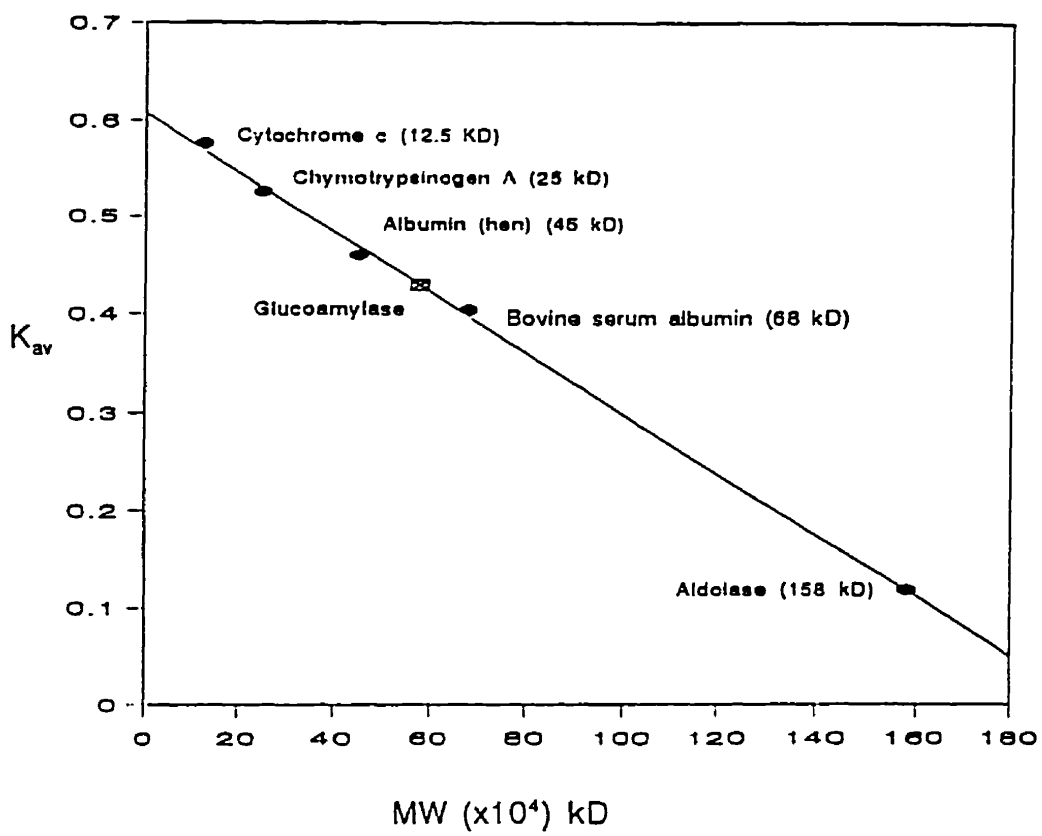


Figure 3.8. Estimation of molecular mass of glucoamylase from *Lactobacillus amylovorus* by gel filtration (two Superose 12 columns arranged in series) using Boehringer gel filtration molecular mass markers. $K_{av} = V_e - V_o = V_t - V_o$, R^2=0.9884, Y = 0.6073 - 3.0966E-06 X.

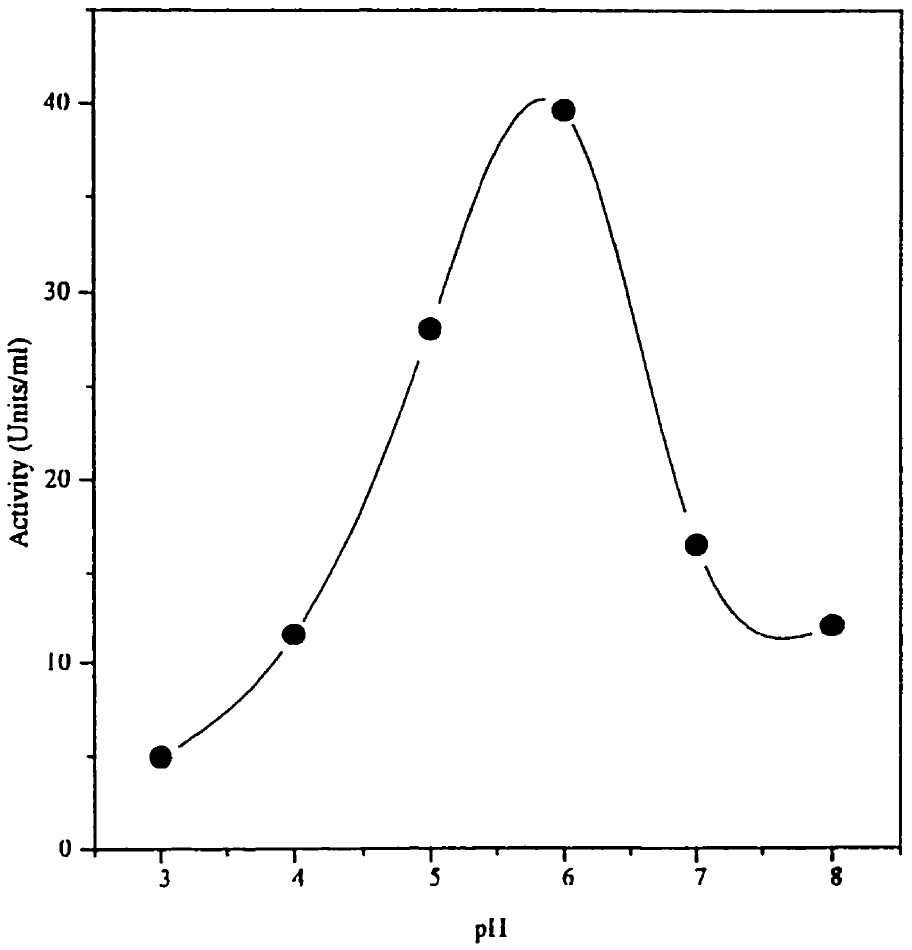


Figure 3.9. Effect of pH on glucoamylase activity of *Lactobacillus amylovorus*.

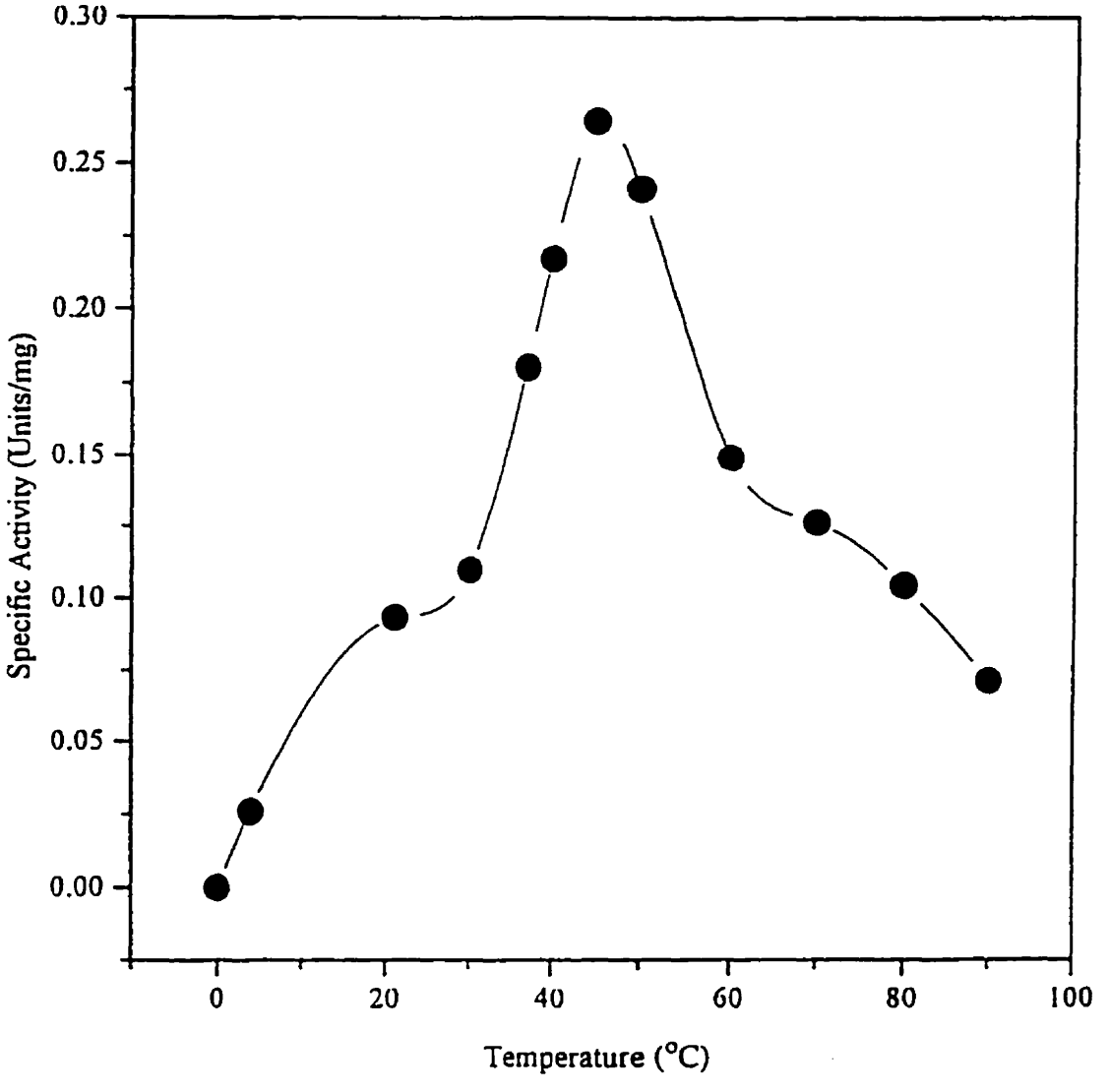


Figure 3.10. Effect of temperature on glucoamylase activity of *Lactobacillus amylovorus*.

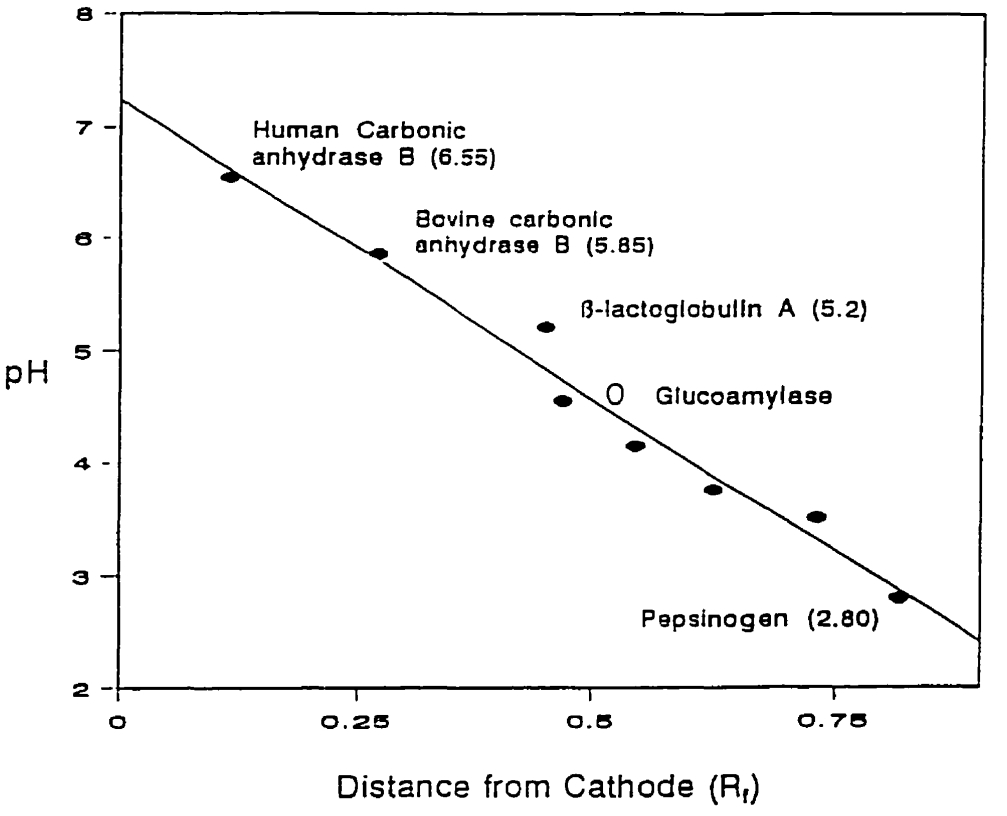


Figure 3.11. Estimation of isoelectric point (pI) of native glucoamylase from *Lactobacillus amylovorus* using a low pI calibration kit (pH 2.5-6.5), R^2=0.9899, Y = 1.33 - 0.04 X.

CHAPTER 4.0

CHARACTERIZATION OF GLUCOAMYLASE FROM *LACTOBACILLUS AMYLOVORUS* ATCC 33621.

In this chapter, the purified glucoamylase from previous work was used for characterization studies. It was necessary to look at properties of the enzyme like kinetic constants, reaction with oligosaccharides of varying chain length, the effect of the end product, glucose, on enzyme action, and also the effect of cations and inhibitors on enzyme activity. The characterization of glucoamylase from *Lactobacillus amylovorus*, added to the knowledge of this enzyme and helped to confirm its potential for applications in the food industry.

The key results of this chapter were summarized in a manuscript for publication in the journal, "Biotechnology Letters". The manuscript entitled "Characterization of glucoamylase from *Lactobacillus amylovorus*" was co-authored by Jennylynd A. James and Byong H. Lee. The experimental work was supervised by Dr. Byong Lee and carried out by Jennylynd James. Jacqueline Beaupré assisted by conducting the HPLC analyses of glucoamylase degradation products. The manuscript was written by Jennylynd James and edited by the supervisor, Dr. Byong Lee prior to submitting it for publication.

4.1 ABSTRACT

An intracellular glucoamylase, purified from *Lactobacillus amylovorus* was found to react selectively on large oligosaccharides. The effect of chain length on enzyme activity was determined using maltose, maltotriose, amylose, amylopectin, dextrin and starch. Kinetic studies indicated low affinity for maltose and maltotriose (K_m 58 g/ml and 178 g/ml) and higher affinity for starch and dextrin (K_m 0.01 g/ml and 0.02 g/ml). HPLC analyses of glucoamylase digestions of starch and dextrin showed that glucose was the main end product of the enzyme action. However, glucoamylase was inhibited almost 50% by 10 mM glucose in solution. The enzyme retained 95% relative activity with α-cyclodextrin which is a rare property, while it possessed only 10% relative activity with cellobiose. Cu^{2+} and Pb^{2+} inhibited glucoamylase at 1.0 mM final concentration but EDTA and other metal chelators had no effect on the enzyme activity. Acarbose (1 mM) and Tris (1 mM) inhibited the enzyme by 84% and 98%, respectively. Iodoacetate and p-chloromecuribenzoic acid caused significant inhibition (98% and 78% respectively) of enzyme activity at 10 mM final concentration. The purified enzyme was found to be thermolabile compared to other bacterial glucoamylases since storage at temperatures greater than 55°C rapidly decreased the enzyme activity. Glucoamylase from *Lb. amylovorus* thus has a good potential for application in the brewing industry.

4.2 INTRODUCTION

The saccharifying ability of glucoamylases has been useful in many important applications in the food industry. Glucoamylase (EC 3.2.1.3) degrades high molecular mass oligosaccharides into ß-D-glucose by cleaving α-1,4 glycosidic bonds in a consecutive manner. The enzyme is also capable of hydrolysing α-1,6 linkages depending on the size of the substrate and position of the bond (Fogarty, 1983). Glucoamylase has been used mainly in the production of high glucose syrups applied in fermentation or production of crystalline glucose and high fructose corn syrup (Fogarty, 1983). Commercial glucoamylases are prepared from mold strains such as *Aspergillus* (Metwally *et al.*, 1991) and *Rhizopus* (Ashikari *et al.* 1985; Ohnishi *et al.* 1990; Yu and Hang, 1991). These enzymes tend to be thermostable which is suitable for high glucose syrup production where temperatures of 60°C are maintained for over 48 h for enzyme action. In the brewing of low calorie beer, however, a thermolabile glucoamylase which can be readily inactivated by pasteurization conditions (60°C for 5.6 min), is required in order to ensure product stability during storage (Lewis and Young, 1995).

Studies of bacterial glucoamylases have been conducted on *Clostridium* sp. (Hyun and Zeikus, 1985; Ohnishi *et al.*, 1991; Specka *et al.*, 1991) and *Halobacterium sodomense* (Chaga *et al.*, 1993). No information has yet been published on the characterization of glucoamylase from a food-grade bacteria in

order to ascertain its suitability for application in the brewing of low calorie beer. In previous studies, an intracellular, thermolabile glucoamylase was identified in *Lactobacillus amylovorus* (James and Lee, 1995). The objectives of this study were to characterize glucoamylase from *Lb.* amylovorus to better understand its industrial potential. The enzyme reaction with oligosaccharides of varying chain length was investigated and substrate specificity, inhibition and kinetics were studied. The thermostability of glucoamylase from *Lb. amylovorus* was also investigated at several temperatures to predict enzyme action at the main stages of the brewing process.

Properties of glucoamylase from *Lb. amylovorus* suggest that the enzyme could be employed in the brewing of low calorie beer. The enzyme was labile at temperatures over 55°C, however, after storage for 10 min at 60°C the enzyme was not completely deactivated. Further studies are required to clone the gene coding for glucoamylase and to characterize the recombinant enzyme in order to evaluate the potential application of this enzyme in the brewing industry.

4.3 MATERIALS AND METHODS

4.3.1 Organism and preparation of purified enzyme

Lactobacillus amylovorus ATCC 33621 obtained from American Type Culture Collection (Maryland, U.S.A.) was activated by two successive transfers

in MRS (de Man *et al.* 1960) broth (Difco Laboratories, Detroit, MI.) and maintained in a sterile broth/glycerol (60%:40%, v/v) solution at -40°C. The preparation of crude cell-free extracts was performed as previously described (James and Lee, 1995). Glucoamylase from *Lb. amylovorus* was purified using a Fast Protein Liquid Chromatography (FPLC) System (Pharmacia Biotech, Baie D'Urfé, Québec, Canada) and Preparative Gel Electrophoresis (BioRad, Mississauga, Ontario, Canada).

4.3.2 Chemicals, reagents and equipment

Unless otherwise specified, all reagents were purchased from Sigma Chemical Company (St. Louis, Mo., U.S.A.). The glucoamylase inhibitor, acarbose was kindly supplied by Drs. E. Möller and M. Mardin of Bayer AG., Germany. All other chemicals were of analytical reagent grade. Analysis of end products of glucoamylase action was conducted on a High Protein Liquid Chromatography (HPLC) System (Waters Chromatography Division, Mississauga, Ontario, Canada) using an ion exclusion, Ion-300 column (300 mm x 20 mm) (Mandel Scientific Co., Rockford, Ontario, Canada).

4.3.3 Enzyme Assays

Glucoamylase activity was determined spectrophotometrically by a

modification of the method of McCleary *et al* (1991). A 100 µl portion of enzyme solution was added to 100 µl of *p*-nitrophenyl-ß-maltoside solution (Megazyme, N.S.W, Australia). The mixture was incubated at 40°C for 10 min and terminated with color developed by the addition of 1.5 ml of 2% Trizma base (Sigma). Enzyme activity was calculated as follows: Activity (Units/ml) = $\blacktriangle A_{410}/t$ x a/b x 1/17.8 x Dilution Factor, where $\blacktriangle A_{410}$ = Absorbance (reaction) - Absorbance (blank), t = Incubation time, a = Final reaction volume, b = Volume of enzyme and 17.8 = $\varepsilon_m$mol *p*-nitrophenol in 2% Trizma base. One unit of enzyme activity was defined as the amount of enzyme required to release one µmole of *p*-nitrophenol from the substrate per min under the defined assay conditions. Specific activity was defined as the number of enzyme units per mg protein.

For measurement of pH and temperature effects on enzyme activity, an alternative substrate, starch was used. Glucoamylase activity was determined by a modification of the method of Ghosh *et al* (1990). A 100 µl aliquot of cell extract was incubated with 100 µl of 1% (w/v) soluble starch (Sigma) in 0.05 M sodium phosphate buffer (pH 6.0) at 45°C for 30 min. The reaction was terminated by immersing the tube in a boiling water bath for 5 min. The glucose liberated by enzymatic action was measured by a glucose oxidase method (Glucose-Trinder test kit, Sigma), using glucose standards (Sigma) for calibration. One unit of glucoamylase was defined as the amount of enzyme required to release 1 µmol/l of glucose per min under the assay conditions and specific activity was defined as the number of enzyme units per mg protein.

4.3.4 Protein assay

Protein was determined spectrophotometrically by the Bicinchoninic Acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as a standard (Smith *et al.* 1985). The method is based on the reaction of Cu^{2+} with peptides in alkaline solution to form Cu^{+} ions which are detected by a highly sensitive and selective BCA reagent. A 0.1 ml sample of enzyme solution was added to 2.0 ml of the assay mixture and incubated at 37°C for 30 min. The absorbance was measured at 562 nm.

4.3.5 Substrate Specificity

Glucoamylase activity on various carbon substrates was determined as outlined by Campos and Felix (1995). Each substrate was dissolved in a 1% (w/v) solution in 0.05 M sodium phosphate buffer at pH 6.0. Reactions with large oligosaccharides, starch and dextrin (1% w/v) were compared with those of smaller oligosaccharides: amylopectin, amylose, maltotriose and maltose (1% w/v) to investigate the effect of carbon chain length. The reactivity of glucoamylase (0.0536 U/mg) with cyclic oligosaccharides was investigated by reaction with α-cyclodextrin and specificity for α-(1,4)-anomers was investigated by reaction with ß-(D)-cellobiose, a ß-(1,4)-anomer.

4.3.6 Enzyme Kinetics

Initial rates of dextrin and starch hydrolysis at 45°C and pH 6.0 in 0.05 M sodium phosphate buffer were determined at various substrate concentrations (1% - 40%) with 3.5 µg of enzyme. A volume of 100 µl of enzyme solution was incubated with 100 µl of substrate and the glucose liberated was measured using the Glucose-Trinder test kit (Sigma) as outlined previously. The apparent kinetic constants K_m and V_{max}, were estimated by the method of Lineweaver and Burk (1934), from the slope and intercept of the regression curve of the inverse of initial velocity (1/V) versus the inverse of substrate concentration (1/S). Apparent kinetic constants for starch, maltose, maltotriose, and amylopectin were estimated under similar conditions to determine the effect of carbon chain length on the kinetic constants.

4.3.7 Products of glucoamylase action and glucose inhibition

The products of glucoamylase action were prepared by incubating the enzyme with 1% (w/v) starch and dextrin in 0.05 M sodium acetate buffer (pH 4.5) 45°C overnight. The end products of glucoamylase and other starch degrading enzymes in crude cell extract were compared to the product(s) of digestion with those of the purified enzyme. Crude enzyme was used at specific activity of 0.0067 units/mg and purified enzyme at 0.0536 units/mg. 500 µl of enzyme was incubated

with 500 µl of substrate solution. The reaction was terminated by boiling in water for 5 min. The mixture was centrifuged at 10,000 g for 15 min using an IEC Centra (Model MP4R, International Equipment Company, Needham Hts., MA, U.S.A.) to remove any precipitates and the supernatant was diluted to the appropriate concentration for detection.

An Ion 300 Interactive ion exclusion column (Mandel Scientific Co.) was used to analyze the oligosaccharides and sugars present in solution before and after enzyme action. This was attached to the Waters HPLC System, Model 600E. The system was equipped with a Waters U6K pump, an automatic sampler (Waters 700, Satellite WISP), a refractive index monitor (model R401), and a module control unit using the Millennium 2010 data processing software (Waters). The flow rate was adjusted to 0.5 ml/min at 25°C using 0.01 M H_2SO_4 as the mobile phase.

Since glucose is the sole end product of glucoamylase action, the effect of varying concentrations of glucose on glucoamylase activity in crude extract and in the purified enzyme was investigated. Glucose (Sigma) at final concentrations of 10, 50, 100, 200, 300, 400 and 500 mM, was incubated with enzyme solution (0.0536 U/mg) for 5 min. Glucoamylase activity was then determined using p-nitrophenyl-ß-maltoside (Megazyme) as outlined previously.

4.3.8 Effect of divalent cations, metal chelators and other inhibitors

The effect of cations on glucoamylase activity was investigated by incubating pure enzyme with Cu^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, K^{+}, Ca^{2+} and Zn^{2+} ions at final concentrations of 0.1 mM, 0.5 mM and 1.0 mM, for 5 min at 40°C. The reaction was initiated by adding an equal volume of p-nitrophenyl-ß-maltoside (Megazyme) and the yellow color developed was measured spectrophotometrically. Inhibition was expressed as a percentage of the activity without effector (control). The effect of the following inhibitors: EDTA, dithiothreitol (DTT), PMSF, Tris, acarbose, deoxynojirimycin, iodoacetate, 1,10 phenanthroline, guanidine/HCl, p-CMB, hydroxyquinoline and mercaptoethanol, on glucoamylase activity was determined at final concentrations of 1.0, 5.0 and 10 mM in the enzyme solution (0.05 M sodium phosphate buffer, pH 6.0).

4.3.9 Effect of storage temperature

To estimate the thermal stability of the enzyme, glucoamylase (0.0536 U/mg) was incubated at 21°C, 37°C, 45°C, 50°C, 55°C, 60°C, 70°C and 80°C and activity was monitored at 10 min intervals for up to 2 h, assaying for residual glucoamylase activity as described previously.

4.4 RESULTS

4.4.1 Substrate specificity and enzyme kinetics

When glucoamylase was incubated with oligosaccharides of varying length, the trend observed was a preference for long chain oligosaccharides. As seen in Table 4.1, the relative activity decreased with a decrease in chain length from starch (100%) to maltose (52%). Glucoamylase reacted 95% with α-cyclodextrin, but activity with D(+)-cellobiose was very low (10%).

Kinetic constants for glucoamylase action on oligosaccharides of varying length, were estimated by constructing Lineweaver-Burk plots from the initial velocity of enzyme reactions (Fig. 4.1). The results in Table 4.2 show that the K_m value for starch (10.4 mg/ml) was lower than the K_m for maltose (58.3 g/ml).

4.4.2 Products of glucoamylase action and glucose inhibition

Figure 4.2 shows the HPLC chromatogram of the products formed by the reaction of crude and purified glucoamylase on starch. Glucose peaks with retention times of 12.7 ± 0.04 min for crude and pure enzyme hydrolysates were detected. Similar chromatograms were obtained in the reaction of glucoamylase with dextrin where glucose peaks also emerged at 12.7 ± 0.04 min (Fig. 4.3). These glucose peaks corresponded to the retention time obtained using a glucose

standard under the same conditions. A large peak corresponding to disaccharides, and peaks for other intermediate oligosaccharides were also obtained in chromatograms of all the hydrolysates.

Relative activity of crude glucoamylase decreased from 30% to 83% when glucose was present at final concentrations of 10 mM to 500 mM (Table 4.3). Purified enzyme was inhibited by even lower glucose concentrations. Inhibition was about 50% at 10 mM final concentration with almost complete inhibition (97%) at 500 mM.

4.4.3 Effect of metals and inhibitors

The reactions with various monovalent and divalent cations showed that Ca^{2+} and Mg^{2+}, had no effect on enzyme activity at 1.0 mM, while Pb^{2+} and Cu^{2+} inhibited activity at 1.0 mM by 76% and 89%, respectively (Fig. 4.4). The metal chelator, EDTA, enhanced enzyme activity up to 50%, at a final concentration of 10 mM. However, the enzyme was strongly inhibited by oligosaccharide analogs deoxynojirimicin (54%) and acarbose (94%) at 10 mM final concentration (Fig. 4.5). Almost complete inhibition of glucoamylase was obtained with Tris and iodoacetate (98%) at 10 mM final concentration.

4.4.4 Effects of storage temperature

Glucoamylase activity remained fairly stable at temperatures between 21°C and 50°C within a 2 h period, but at temperatures above 55°C enzyme activity was lost rapidly (Fig. 4.6). At 60°C glucoamylase activity decreased almost 95% within a 10 min period.

4.5 DISCUSSION

Glucoamylase reacts selectively with large chain oligosaccharides like dextrin. Thus, the addition of this enzyme is favorable in the conversion of maltodextrins to fermentable sugars during the mashing or fermentation stages of brewing (Dougherty, 1977; Lewis and Young, 1995). Increase in specific saccharifying activity of glucoamylase from *Lb. amylovorus* correlated with an increase in the number of glucose residues present in the oligosaccharides. This indicated that the binding of the substrate was facilitated by the length of the oligosaccharide chain, a behavior typical of glucoamylases produced by mold strains (Campos and Felix, 1995). This observation was further supported by the fact that there was a decrease in K_m with longer substrate chain length. Glucoamylase from *Aspergillus awamori* wild type (Sierks and Svensson, 1993) and *Humicola grisea* (Campos and Felix, 1995) showed the same specificity based on length of substrate. This property would favor addition of glucoamylase for the

conversion of maltodextrins to fermentable sugars during the mashing or fermentation stages of brewing (Dougherty, 1977; Lewis and Young, 1995).

The specificity of glucoamylase for α-1,4 anomers was confirmed by its inability to hydrolyze ß-(D)-cellobiose which has ß-(1,4) linkages. However, glucoamylase from *Lb. amylovorus* was surprisingly able to degrade α-cyclodextrin which is contrary to the rule of glucoamylase exoaction from the non-reducing chain end of oligosaccharides. A glucoamylase isolated from a *Flavobacterium* sp. also displayed this rare ability to react with cyclic oligosaccharides (Bender, 1981). This enzyme was also remarkably similar to glucoamylase from *Lb. amylovorus*, in that it was active in the pH range 5.5 to 6.5 and was heat sensitive above 55°C (Fogarty, 1983; James and Lee, 1995).

Glucose was revealed as the main end product of enzyme action after hydrolysis of starch and dextrin at 45°C overnight. A strong glucose peak appeared in the HPLC chromatograms of both crude and purified enzyme digests of starch (Fig. 4.2) and dextrin (Fig. 4.3), confirming the presence of glucoamylase. Other saccharifying enzymes like α-amylase in crude extract have a synergistic effect on glucoamylase action (Abe *et al.* 1988). α-Amylase acts randomly to cleave internal α-1,4 glycosidic bonds in amylose and amylopectin producing non-reducing chain ends from which glucoamylase would remove glucose units consecutively (Fogarty, 1983). This may account for the larger glucose peak resulting from crude enzyme digestion rather than pure enzyme digestion.

Oligosaccharide peaks were obtained in the chromatograms and this

indicates that starch and dextrin digestions were not complete. A longer incubation time may have been needed for complete oligosaccharide hydrolysis, however, the presence of transglucosidase in crude enzyme, may be responsible for the formation of small oligosaccharides by catalysing reversion reactions (Fogarty, 1983). The inhibition by the end product, glucose, may have slowed down the action of glucoamylase. The chromatograms for dextrin and starch showed similar oligosaccharide profiles since starch was broken down to dextrins which were then cleaved in an identical manner.

The inhibition by glucose was determined at different concentrations. Crude enzyme was inhibited about 50% at 50 mM, while purified enzyme was inhibited 50% at 10 mM. The protective effect of other proteins in the crude extract could account for the lower inhibition of glucoamylase in the crude extract. The glucose inhibition of glucoamylase at greater than 10 mM, may be one hindrance in the use of this enzyme in brewing, if the reducing sugar is not promptly fermented into alcohol. Although most glucoamylases are sensitive to glucose at high concentrations, glucoamylase of *Humicola grisea* has been found to be relatively insensitive to glucose concentrations up to 200 mM, retaining 65% activity (Campos and Felix, 1995).

There was no requirement of metal ions for enzyme reaction as is the case with α-amylase which has enhanced enzyme activity with Ca^{2+} and Mg^{2+} (Bernstein and Willox, 1977; Vihinen and Mäntsälä, 1989). However, glucoamylase from *Lb. amylovorus* is inhibited by Pb^{2+} and Cu^{2+}. The presence of Pb^{2+} or Cu^{2+} in the

water used for brewing, would therefore significantly inhibit enzyme activity, reducing the saccharification of maltodextrins (Lewis and Young, 1995). Cu^{2+} may be found in old breweries which still use copper containers and reactors, for example in Brazil.

The metal chelator, EDTA had no inhibitory effect on enzyme activity. Instead, it was found to enhance activity up to 50%, possibly by reacting with certain activators in this solution. The oligosaccharide analogs acarbose and deoxynojirymicyn both inhibited glucoamylase activity significantly. Acarbose, a pseudotetrasaccharide, is the most effective known inhibitor of glucoamylase, having a $K_d \approx 10^{-12}$ M (Svensson and Sierks, 1992). The inhibitor contains a valineamine residue at its "non-reducing" end and 4-amino-4,6-dideoxy-α-D-glucopyranose in the place of the second glucose residue (Aleshin *et al.* 1994). 1-Deoxynojirimycin is a single residue inhibitor of glucoamylase with K_i= 96 μM (Harris *et al.* 1993). These compounds have been used to study the mechanisms of hydrolysis of substrates with glucoamylase.

The amino alcohol, Tris, almost completely inhibited glucoamylase activity at 10 mM final concentration. Similar inhibition was observed in glucoamylases from *Penicillium* sp. and *Mucor* sp. (Vihinen and Mäntsälä, 1989) and enzymes like α-glucosidase with similar activity to glucoamylases (Shantha Kumara *et al.* 1993). Glucoamylase was inhibited 78% by p-chloromecuribenzoic acid at 10 mM. Inhibition by other sulfhydryl inhibitors like iodoacetate, indicates the presence of a sulfhydryl group near the enzyme active site, and its involvement in catalysis.

Glucoamylases from some *Aspergillus* strains were also inhibited by p-hydroxymecuribenzoic acid and exhibited similar inhibition by Cu^{2+} or activation by EDTA (Vihinen and Mäntsälä, 1989). The inhibitory studies on crude enzyme showed similar results (James and Lee, 1995).

Glucoamylase from *Lb. amylovorus* was labile at temperatures above 55°C. This is contrary to glucoamylase from *Clostridium thermohydrosulfuricum*, *Humicola lanuginosa*, *Thermomyces lanuginosus* and *Humicola grisea*, which are considered thermostable (Fogarty, 1983; Vihinen and Mäntsälä, 1989; Campos and Felix, 1995). Thus, pasteurization at 60°C should be sufficient to destroy the enzyme activity at the final stage of brewing.

4.6 CONCLUSION

The properties of glucoamylase from *Lactobacillus amylovorus* suggest that the enzyme could be employed in the brewing of low calorie beer. The low K_m for dextrin would be beneficial in the hydrolysis of dextrins from malted barley, and the low thermostability allow for heat inactivation before bottling of the final product. However, the sensitivity of the enzyme to glucose would be a problem if free glucose were allowed to accumulate above 10 mM concentration without being fermented by brewer's yeast. The gene coding for this glucoamylase from *Lb. amylovorus* should be cloned and the recombinant enzyme characterized to determine if the enzyme properties remain the same. Cloning of the glucoamylase

gene in brewer's yeast would minimize the need to add commercial glucoamylases in low calorie beer production, thus lowering overall production costs.

Table 4.1. Substrate specificity of glucoamylase from *Lactobacillus amylovorus*.

Substrate	Relative Activity (% ± SD)
Starch	100
Potato Dextrin	84 ± 1.06
Maltodextrin DE10	68 ± 3.19
Amylopectin	59 ± 2.13
Amylose	75 ± 1.85
Maltotriose	60 ± 1.50
Maltose	52 ± 1.06
α-Cyclodextrin	95 ± 0.79
D(+)-Cellobiose	10 ± 3.19

Table 4.2. Enzyme kinetic constants for the reaction of purified glucoamylase from *Lactobacillus amylovorus* with various oligosaccharides.

Substrate	K_m (g/ml)	V_{max} (mM $ml^{-1}min^{-1}$)	Correlation Coefficient (R^2)
Starch	0.01	0.01	0.83
Potato Dextrin	0.02	0.14	0.92
Amylopectin	2.02	9.33	0.97
Maltose	58.31	1.84	0.98
Maltotriose	177.88	0.73	0.98

Table 4.3. Effect of glucose concentration on glucoamylase activity in crude extract and purified glucoamylase from *Lactobacillus amylovorus.*

	Relative Activity (%)	
Glucose concentration (mM)	Crude Extract	Purified Glucoamylase
500	16.62 ± 0.05	3.37 ± 0.08
400	20.64 ± 1.46	4.86 ± 0.00
300	20.23 ± 0.07	7.47 ± 0.29
200	28.77 ± 1.13	9.25 ± 0.66
100	34.42 ± 0.44	18.68 ± 0.34
50	51.07 ± 0.04	21.61 ± 0.27
10	70.61 ± 2.94	48.88 ± 1.31
0	100	100

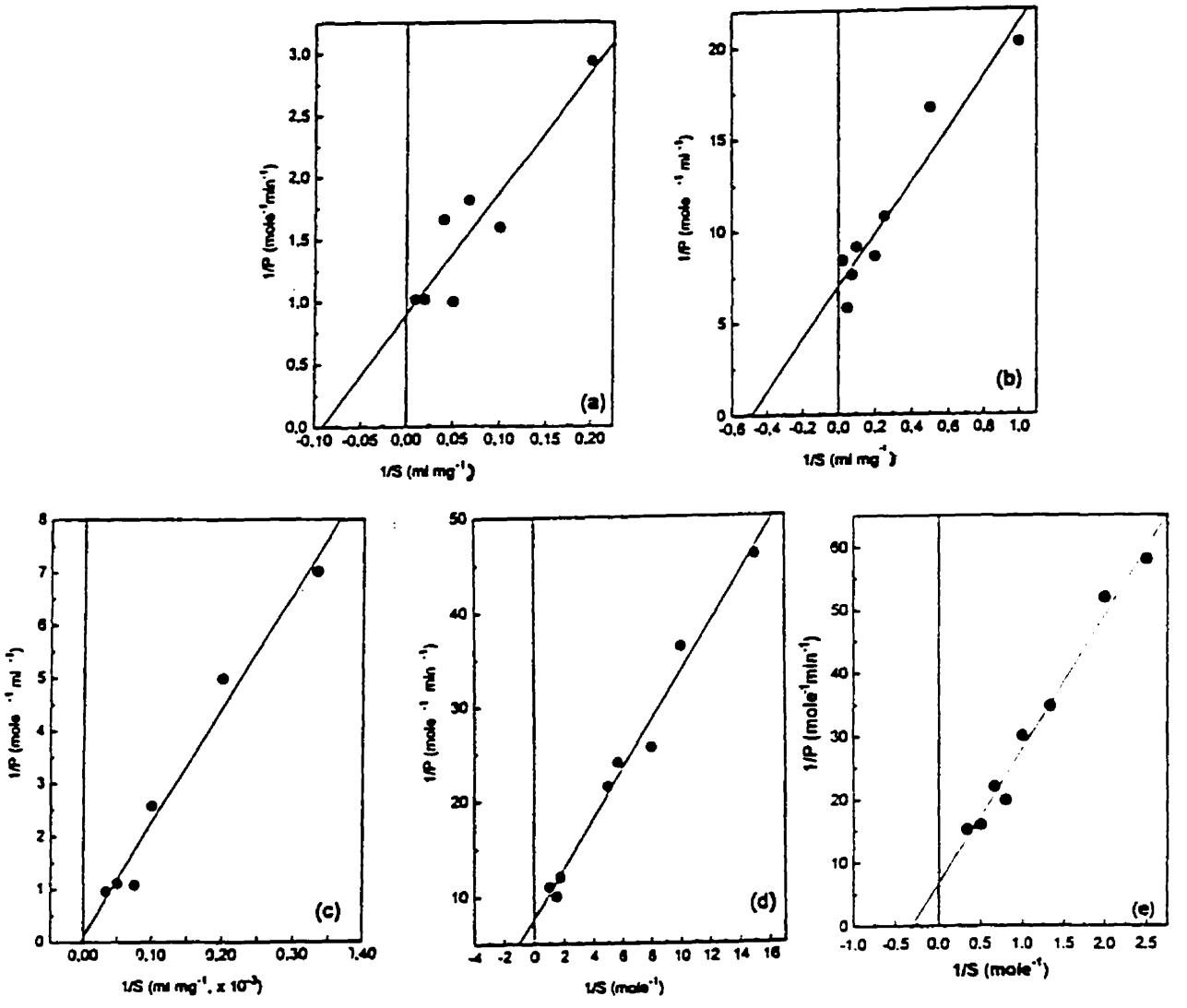


Figure 4.1. Lineweaver-Burk plots of glucoamylase activity of *Lb. amylovorus* with the substrates (a) starch, R^2 = 0.8321 , Y = 11.6364 + 9.0997X; (b) dextrin, R^2 = 0.9159, Y = 6.9892 + 14.3107X; (c) amylopectin, R^2 = 0.9710, Y = 0.1072 + 21.6039X; (d) maltotriose, R^2 = 0.9782, Y = 3.0096 + 23.8511X; and (e) maltose, R^2 = 0.9801, Y = 7.6104 + 2.6837X; in 0.05 mol l^{-1} sodium phosphate buffer, pH 6.0.

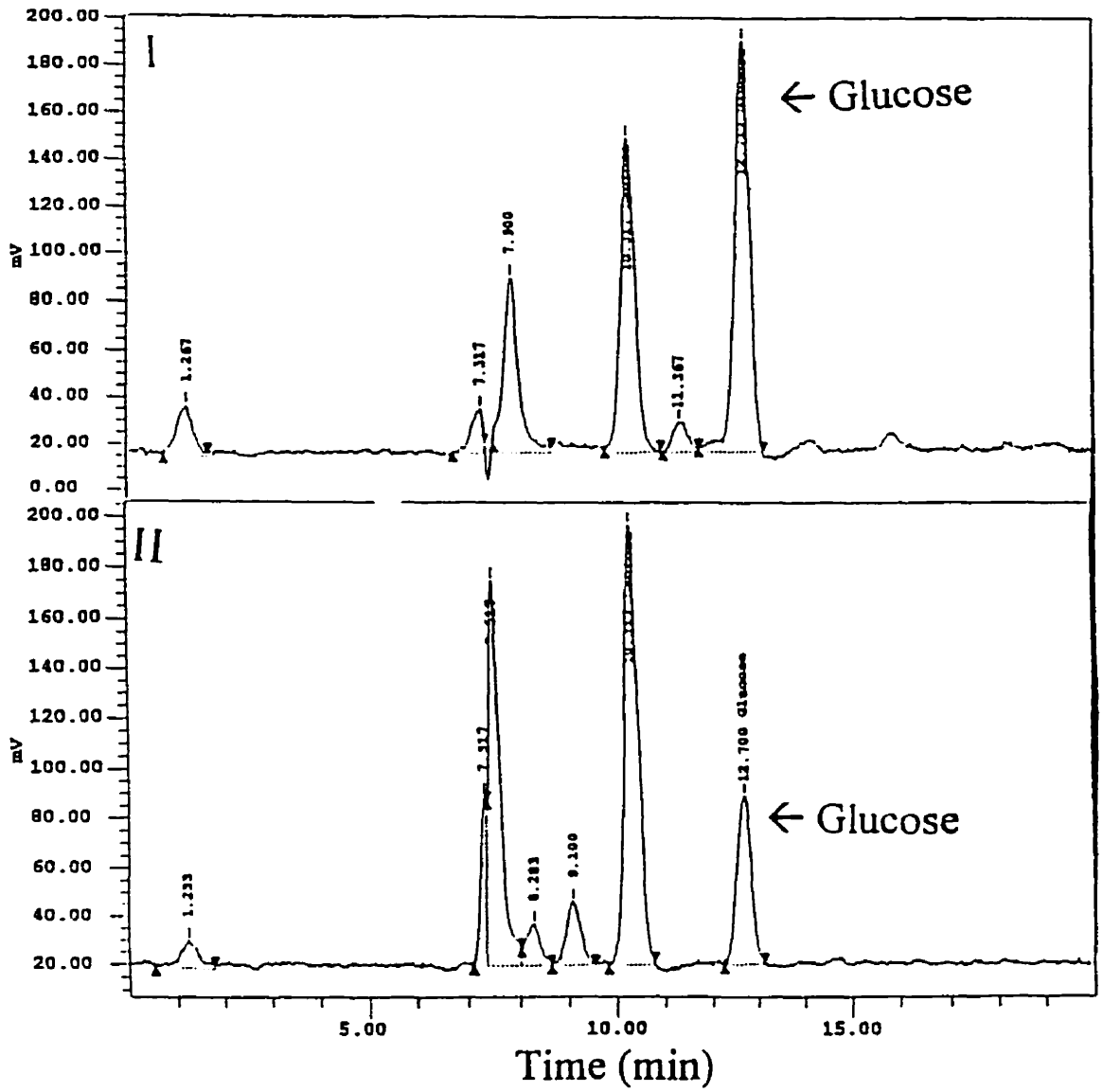


Figure 4.2. HPLC Chromatogram (Ion 300 column) of the oligosaccharide products of glucoamylase degradation of starch at 45°C (I, Crude Enzyme; II, Pure Enzyme)

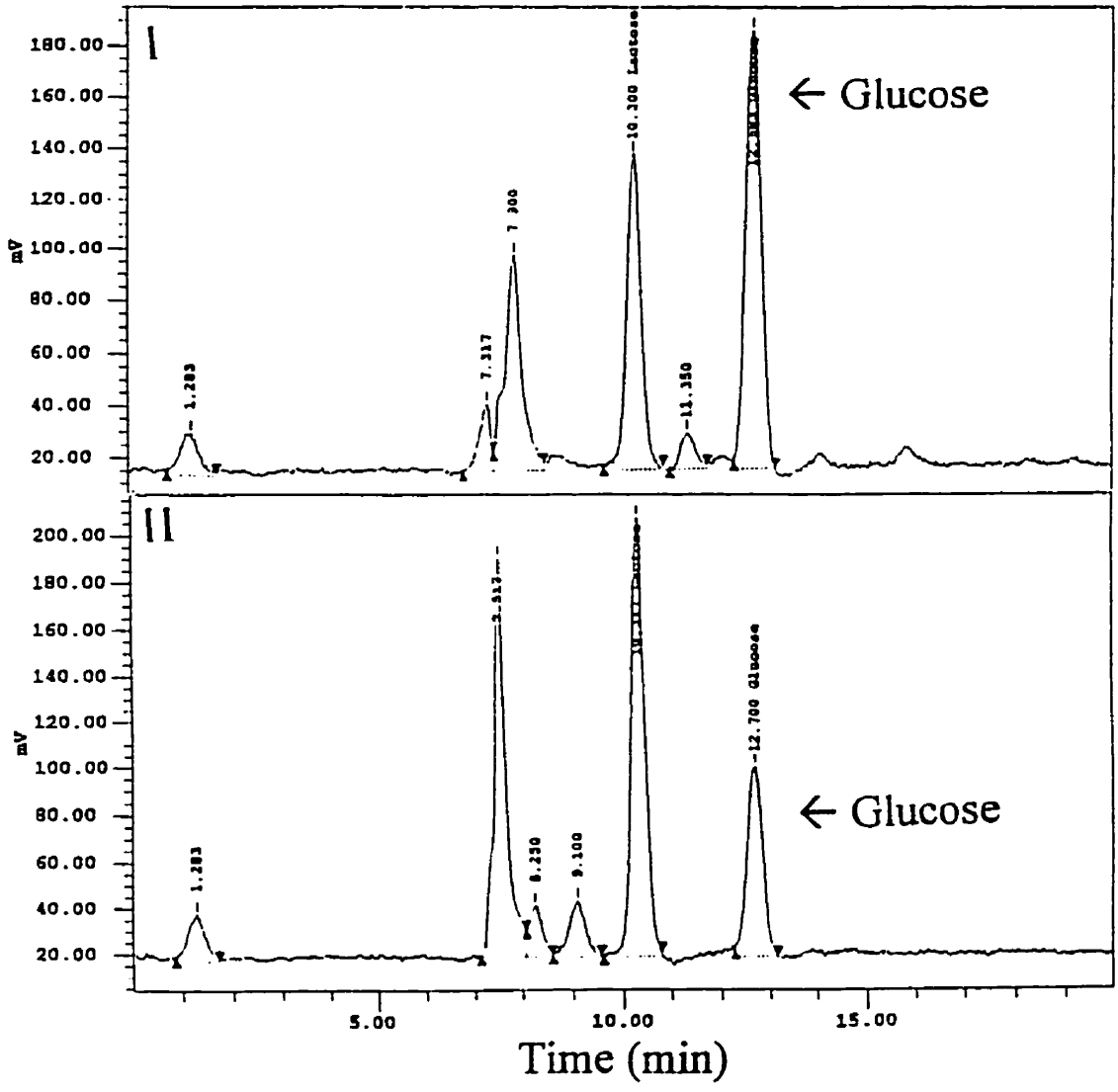


Figure 4.3. HPLC Chromatogram (Ion 300 column) of the oligosaccharide products of glucoamylase degradation of dextrin at 45°C (I, Crude Enzyme; II, Pure Enzyme)

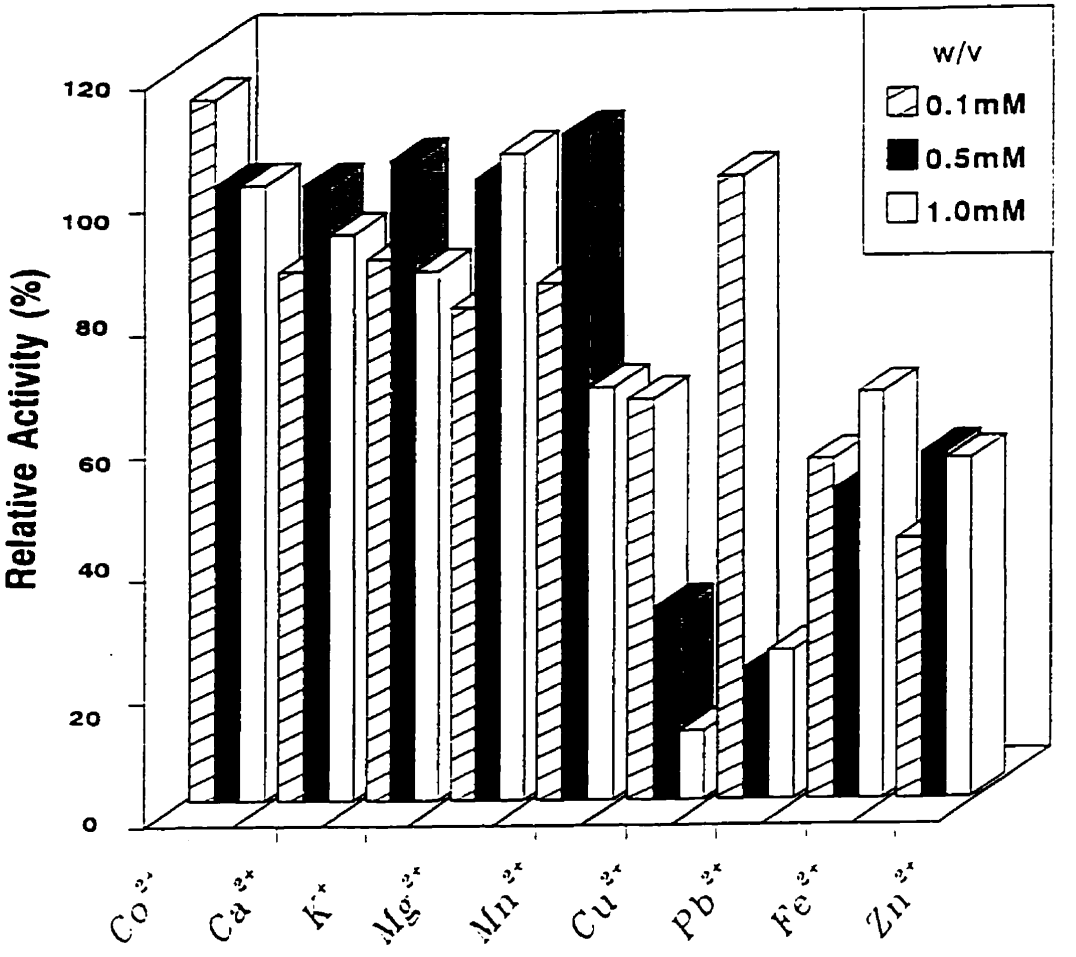


Figure 4.4. Effect of cations on glucoamylase activity of *Lactobacillus amylovorus*.

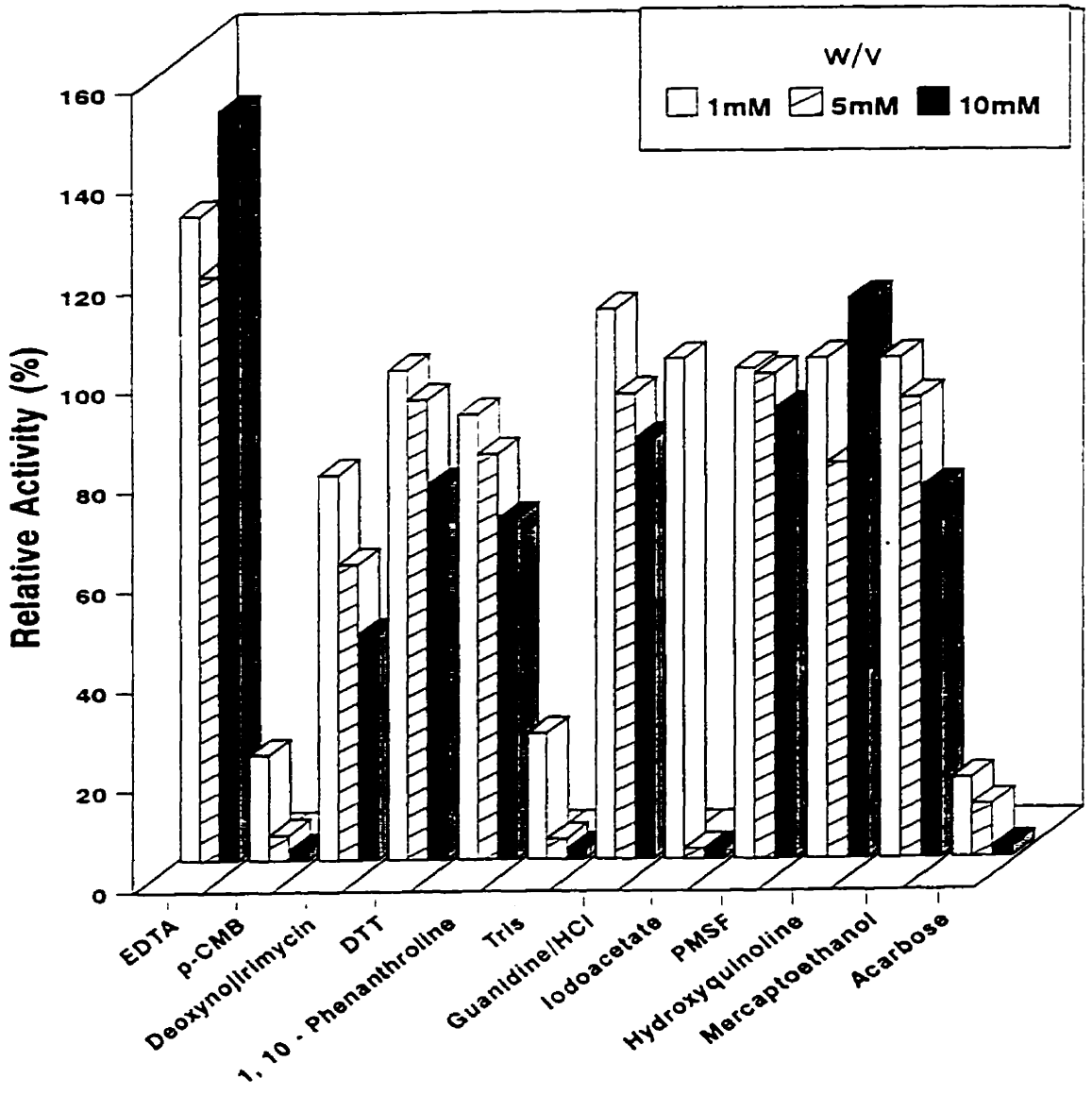


Figure 4.5. Effect of inhibitors, metal chelators and other substances on glucoamylase activity of *Lactobacillus amylovorus*.

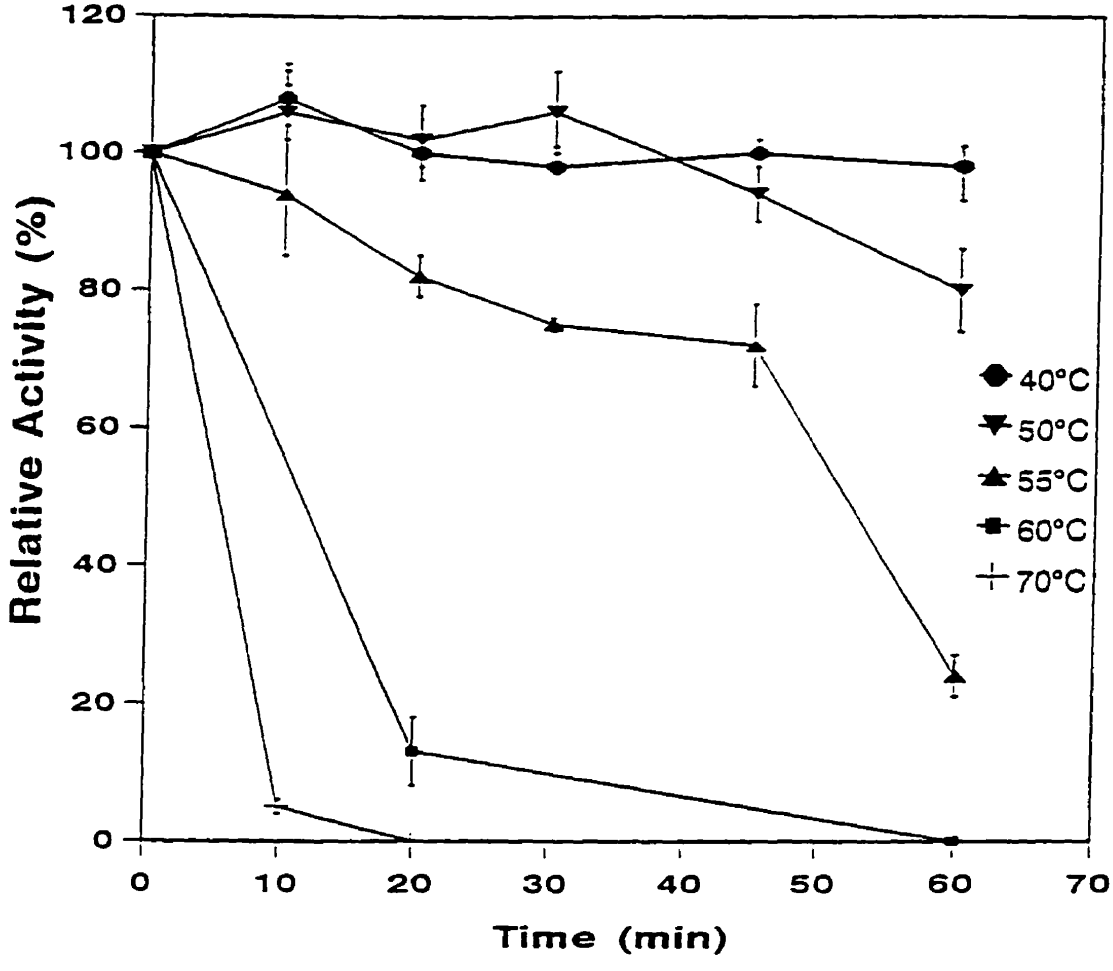


Figure 4.6. Effect of storage temperature on glucoamylase activity of *Lactobacillus amylovorus*.

CHAPTER 5

CLONING AND EXPRESSION OF A GLUCOAMYLASE GENE FROM *LACTOBACILLUS AMYLOVORUS* ATCC 33621 IN *ESCHERICHIA COLI*

Although the properties of glucoamylase were proven to be beneficial for the brewing industry, the expensive process of purifying an intracellular glucoamylase from *Lactobacillus amylovorus*, would not make this enzyme competitive for application in the brewing industry. The enzyme's potential could be best assessed by the cloning of this glucoamylase gene first in *Escherichia coli*, then in *Saccharomyces cerevisiae*. In this chapter the cloning of the glucoamylase gene from *Lactobacillus amylovorus* in *Escherichia coli* is described. The cell extract of recombinant clones was assayed for glucoamylase activity and the recombinant enzyme activity was compared to that of the wild-type enzyme.

The main results of the cloning experiments were submitted as a manuscript for publication in the journal, "Biotechnology Letters". The manuscript entitled "Cloning of glucoamylase from *Lactobacillus amylovorus*" was co-authored by Jennylynd A. James, Normand Robert and Byong H. Lee. The experimental work was supervised by Dr. Byong Lee and Normand Robert. Normand Robert developed several experimental protocols and did the actual training in genetic engineering techniques. Experimental work and the writing of the manuscript were carried out by Jennylynd James. The manuscript was edited by Dr. Byong Lee prior to submitting it for publication.

5.1 ABSTRACT

The glucoamylase gene from *Lactobacillus amylovorus* was cloned and expressed in *Escherichia coli.* A genomic DNA library from *Lactobacillus amylovorus* was prepared by partially digesting genomic DNA with the restriction enzyme, *Eco*RI, and ligating random fragments to the *Eco*RI digested cloning vector pZErO-1.1. Transformants were first screened on LB plates containing IPTG, and the antibiotic Zeocin. This was followed by hybridization studies using a digoxigenin labeled probe prepared from the *STA2* glucoamylase gene from *Saccharomyces cerevisiae* var. *diastaticus*. Three putative clones hybridized to the probe were isolated. The physical maps and restriction map of the recombinant plasmids were constructed. These plasmids designated pG1, pG13 and pG35 contained inserts of about 6.4 Kb, 5.2 Kb and 5.9 Kb, respectively. The expression and biochemical properties of glucoamylase from three recombinant clones were compared with those of wild type glucoamylase. The optimum pH and temperature of 6.0 and 45°C, respectively, were equivalent for both recombinant and purified, wild type glucoamylase. Also, the enzymes were found to be thermolabile at temperatures above 55°C.

5.2 INTRODUCTION

The ability of glucoamylase to hydrolyse α-1,4 and α-1,6-glycosidic bonds

(debranching activity) is important for complete starch hydrolysis. As a result, this enzyme finds many applications in the food industry including the degradation of starch into glucose for production of syrups, and whole grain hydrolysis for alcohol production and brewing. *Saccharomyces cerevisiae*, the yeast used in fermentation, can only ferment maltose, maltotriose, sucrose, glucose and fructose (Eratt and Nasim, 1989). Larger sugars and starch must be hydrolysed by enzymes or by the use of heat, before fermentation can proceed. Many researchers have investigated the cloning of fungal glucoamylase genes in *S. cerevisiae*, to produce a yeast which can hydrolyse starch and then ferment the sugars produced (Ashikari *et al.* 1985, Innis *et al.* 1985, Yamashita *et al.* 1985, Pretorius *et al.* 1986, Dohmen *et al.* 1990, Kim *et al.* 1994). Most of these glucoamylases are thermostable and are very important in the production of high fructose corn syrup.

In the brewing industry, however, a heat labile glucoamylase which can be readily inactivated during the final pasteurization step is required. Glucoamylase is sometimes added in the brewing of low calorie beer, in order to hydrolyse complex carbohydrates of wort into fermentable sugars. When thermostable enzymes are used, residual enzyme activity may remain in finished product causing sweetening as the dextrins continue to be hydrolysed. Eratt and Nasim (1989) patented a method for the cloning of a thermolabile glucoamylase gene from *Saccharomyces cerevisiae* var. *diastaticus* (called *S. diastaticus*) into *Saccharomyces cerevisiae*. This glucoamylase was found to be temperature labile

at 50°C and 60°C. Kim *et al.* (1994) constructed self replicating plasmids containing the *STA2* glucoamylase gene of *Saccharomyces cerevisiae* var. *diastaticus* and transformed various brewing strains. Pilot brewing tests with glucoamylase-secreting transformants produced superattenuated beers at accelerated fermentation rates.

The glucoamylase gene of the membrane bound glucoamylase of *Clostridium* sp. G0005 was the first bacterial glucoamylase gene to be cloned (Ohnishi *et al.* 1992). To date, there has been no report of a thermolabile glucoamylase gene being cloned from a food grade bacteria. *Lactobacillus amylovorus* was discovered growing on waste corn (Nakamura, 1981), and this amylolytic bacteria is known to secrete large amounts of α-amylase (Burgess-Cassler and Imam, 1991) and to produce glucoamylase intracellularly (James and Lee, 1995). The objectives of this study were to clone the glucoamylase gene from *Lactobacillus amylovorus* in *Escherichia coli*, and to characterize the recombinant enzyme in terms of its thermostability and effect of pH on enzyme activity. The gene can be further cloned in brewer's yeast for a potential application in brewing, but an effective mechanism must be found to improve gene expression.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial Strains, plasmids and media.

Genomic DNA was obtained from *Lactobacillus amylovorus* ATCC 33621

(American Type Culture Collection, Maryland). This was grown in MRS (de Man *et al.* 1960) medium without dextrose (Institut Rosell Inc., Québec, Canada), supplemented with 1.0% potato dextrin (Sigma Chemical Co., St. Louis, MO), for 18 h at 37°C (James and Lee, 1995). The Zero Background cloning kit (Invitrogen Corp., San Diego, CA) was used to conduct cloning experiments. *Escherichia coli* TOP10F' (Invitrogen) was used as the recipient strain in all transformation experiments. The pZErO-1.1 plasmid vector supplied by Invitrogen was used for preparation of the genomic library. Low salt LB-Zeocin-IPTG medium (LB-Luria Bertani) was prepared for growth and selection of recombinant cells as follows:- 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% bactoagar (Difco Laboratories, Detroit, MI). The pH was adjusted to 7.5 using 5 M NaOH and the solution was autoclaved at 121°C for 15 min. After cooling to ≈55°C, Zeocin (Invitrogen) and IPTG (Sigma) were added to final concentrations of 50 µg/ml and 1 mM, respectively. Positive clones were grown in SOB-Zeocin medium for optimal growth and plasmid yield. SOB-Zeocin medium consisted of 2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 M KCl, 10 mM $MgCl_2$, Zeocin 50µg/ml at pH 7.5.

A non-radioactive oligonucleotide probe was prepared using the plasmid pSA3 which carries the *Saccharomyces diastaticus STA2* glucoamylase gene. This plasmid was generously donated by Prof. Isak S. Pretorius of the University of Stellenbosch, South Africa. The promoter and the structural sequences of *STA2* are carried by the *E. coli*-yeast shuttle vector YEp13, to form the plasmid pSA3 of 19 Kb.

5.3.2 Chemicals and enzymes

Unless otherwise specified, all chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes, ß-agarase, agarose and molecular mass markers, were obtained from Boehringer Mannheim (Laval, Québec, Canada). Other chemicals were obtained from Anachemia (Montreal, Québec, Canada).

5.3.3 Preparation of chromosomal DNA and plasmids

5.3.3.1 Isolation of genomic DNA from Lactobacillus amylovorus

DNA was isolated from *Lactobacillus amylovorus* ATCC 33621 using a modification of the lysozyme-proteinase K method (Mielenz, 1983). Cells were revived by two successive transfers in MRS (de Man *et al.* 1960) broth and maintained in a sterile broth/glycerol (60%:40%) solution at -40°C. Working cultures were prepared by two successive transfers of stock culture to MRS broth at 18 h intervals at 37°C.

A culture of *Lb. amylovorus* (500 ml) was prepared by 1.0 % inoculation of cells in MRS medium without dextrose, supplemented with 1.0% potato dextrin for 18 h at 37°C. Cells were harvested after 18 h by centrifugation at 8,000 g for 15 min at 4°C. Cells were then washed twice by suspension in a solution containing

12% sucrose and Tris (25 mM) at pH 8.0. The pellet was suspended in 10 ml of this solution and digested with 250 µg mutanolysin and 200 mg of lysozyme (Boehringer) for 2 h at 37°C. Then, 1 ml of 10% sodium dodecyl sulfate (SDS), 2 ml 250 mM EDTA were added, and 10 mg of proteinase K (GIBCO BRL Life Technologies Inc., Gathesburg, MD) was added to break down proteins. This mixture was incubated at 37°C for 30 min, and 2.4 ml of 5 M NaCl, 2.03 ml a mixture of a CTAB and NaCl mixture (10%/0.7 M) were added. CTAB was used as a detergent to break up polysaccharides produced by cells during growth. The mixture was incubated at 65°C for 20 min with stirring.

This was followed by two extractions with an equal volume of chloroform/isoamyl alcohol solution (24:1). Isoamylalcohol was used to reduce the foaming caused by the interaction of proteins with chloroform and thus give a cleaner interface. The upper aqueous layer was removed to a sterile tube and DNA was precipitated by adding 0.6 volumes of isopropanol. The process was accelerated by incubation at -70°C for 30 min. Precipitated DNA was centrifuged at 6,000 g for 15 min and the supernatant was removed. The DNA was air dried and dissolved overnight in 500 µl H_2O.

Further purification of DNA in solution was carried out to remove any residual proteins present. Solutions of NaCl (5 M, 92 µl) and CTAB/NaCl (5%/0.5 M, 78 µl) were added to the DNA solution. The mixture was incubated for 20 min at 65°C. Protein was then extracted with an equal volume of a chloroform/isoamyl alcohol (24:1) solution. The tube was mixed then microfuged at 10,000 g for 10

min. The aqueous supernatant was transferred to a sterile tube and NaCl was added to a final concentration of 0.5 M. The mixture was microfuged at 10,000 g for 10 min. The supernatant was then removed and 0.6 volumes of ethanol were added to precipitate DNA with incubation at -70°C for 30 min. The DNA formed was collected by centrifugation at 10,000 g for 15 min. The DNA pellet was dissolved in 250 µl H_2O overnight.

5.3.3.2 Plasmid DNA preparation and mini-preparation (mini-prep)

Plasmid DNA for probe preparation, and DNA from recombinant clones, were prepared using a modification of the alkaline lysis method (Maniatis *et al.* 1989; Ausubel *et al.* 1994). *E. coli* cells were grown overnight in 500 ml LB medium at 37°C with shaking. Cells were harvested by centrifugation at 8,000 g for 10 min and washed twice with 100 ml STE buffer (0.1 M NaCl, 10 mM Tris, pH 7.8, 1 mM EDTA). The pellet was suspended thoroughly in 10 ml solution I (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA). To this suspension lysozyme was added to a final concentration of 5 mg/ml. Solution II (0.2 N NaOH and 1% SDS) (20 ml) was added and the mixture was left on ice for 10 min for clear lysing of the cells. Then, 15 ml cold potassium acetate (5 M, pH 4.8) was added and the suspension mixed and left on ice for 10 min. The mixture was centrifuged at 4,500 g for 30 min and the supernatant was filtered through 2 kimwipes to remove cell debris. Isopropanol (0.6 vols.) was added to the supernatant and the mixture was

stored at -70°C for 30 min. The precipitated DNA was pelleted by centrifugation (4,500 g; 20 min), and the pellet was left to air dry.

The plasmid DNA was further purified by dissolving the pellet in 6 ml 1xTE buffer (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0). Phenol (6 ml) was added and the solution was mixed with inversion to remove proteins and other debris. The suspension was centrifuged at 4500 g for 10 min to separate the phases. The aqueous phase containing DNA was removed and again washed with 3 ml chloroform and 3 ml phenol by mixing. The phases were separated by centrifugation at 4,500 g and the aqueous phase was washed in a final step with 6 ml chloroform. DNA was precipitated with 0.6 vol. of cold ethanol and the solution was stored at -70°C for 30 min. The DNA pellet obtained after centrifugation, was left to air dry before being dissolved in 1xTE buffer for further analyses.

Plasmid mini-prep was used to isolate plasmids from the numerous recombinant clones for analyses. The transformants cells were cultivated in 2 ml LB-Zeocin medium at 37°C with shaking overnight. Aliquots (1.5 ml) of each culture were transferred to microfuge tubes and cells were harvested by centrifugation at 10,000 g for 30 sec. The supernatant was discarded and the pellet was suspended in 200 µl STEt buffer (8% Sucrose, 0.5% Triton X100, 50 mM Tris/HCl, pH 8.0). Freshly prepared lysozyme (25 µl, 10 µg/ml) was added to each tube and the mixture was vortexed for 3 sec. The tubes were placed in a boiling water bath for 40 sec for cell lysis and then centrifuged at 10,000 g for 15 min. The pellet was

removed with a toothpick and 500 µl of ethanol was added to each tube to precipitate DNA. The mixture was stored at -70°C for 30 min and DNA was removed by centrifugation at 10,000 g for 15 min. The DNA pellet was air dried and then dissolved in 50 µl H_2O for further analyses.

5.3.4 DNA Standard curve preparation

A DNA standard curve was prepared for estimation of DNA concentration, using a TKO 120 fluorometer (Hoefer Scientific Instruments, San Francisco, California). Calf thymus DNA (Clontech Laboratories, Inc., Palo Alto, California) was used as the standard. Increasing concentrations of calf thymus DNA were mixed with an equal volume of a capillary assay reagent prepared with 10x TNE buffer and Hoeschst 33258 stock solution (Hoefer Scientific Instruments) as outlined in the instructions manual (10xTNE buffer: 100 mM Tris, 10 mM EDTA, 2.0 M NaCl, pH 7.4). Readings were taken in a 10 µl capillary tube which allowed the measurement of small volumes. The scale was adjusted to 110 fluorometric units for 0.1 µg/µl calf thymus DNA to account for the differences in GC content in other DNA samples. A linear regression curve was prepared using the Y axis as TKO units and X axis as Total DNA measured. The curve was then used to solve for unknown DNA concentrations (Fig. 5.1). DNA was also estimated by measuring the optical density of one µl DNA solution dissolved in TE buffer at the UV absorbance wavelength 260 nm. One unit of absorbance is estimated as 50

µg/ml DNA.

5.3.5 Restriction enzyme digests

Lactobacillus amylovorus DNA was digested with the restriction enzyme (RE), *EcoR*I. For every µg of DNA, 10 U of enzyme activity was added. DNA was digested for 2 h at 37°C for complete digestion, or 15 min and 30 min for random, partial digestion. The DNA fragments were then precipitated in two volumes of ethanol and the suspension was incubated at -70°C for 30 min to precipitate DNA. The DNA precipitate was collected by centrifugation at 10,000 g for 15 min and the pellet was air dried. DNA was resuspended in sterile Millipore water and left overnight to dissolve. The DNA digest was run on a 0.7% agarose gel to determine the extent of digestion.

5.3.6 Construction of genomic DNA library of *Lb. amylovorus* in *E. coli*

The Zero Background Cloning Kit (Invitrogen, San Diego, CA), which employs a positive selection mechanism, was used for cloning DNA fragments. The cloning vector, pZErO-1.1, contains the lethal *Escherichia coli* gene, *ccd*B, fused to the C-terminus of *lac*Zα (Bernard *et al.* 1994). When a DNA fragment is introduced at this point, expression of the *lac*Zα-*ccd*B fusion gene is disrupted, permitting growth of only positive recombinants. Cells containing a non-

recombinant vector are killed when induced with IPTG. The vector also contains the Zeocin resistance gene for selection in *E. coli*. Zeocin is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces* (Berdy, 1980). It shows strong toxicity against bacteria, fungi (including yeast), plants and mammalian cell lines, by binding chromosomal DNA and causing random double-stranded breaks. When using pZErO-1.1, no vector dephosphorylation or blue/white color screening with X-gal is required.

Chromosomal DNA from *Lactobacillus amylovorus* was partially digested with *EcoR*I for 15 min and 30 min and the digests were combined. The cloning vector pZErO-1.1 was also cleaved with *EcoR*I, and the rapid DNA ligation kit supplied by Boehringer was used to ligate sticky ended DNA fragments to vector DNA. The kit contained T_4 DNA ligase, DNA dilution buffer and DNA ligation buffer. The principles of DNA ligation were the same as that outlined in Sambrook *et al.* (1989) except that there was no need to dephosphorylate the plasmid vector with alkaline phosphatase.

Competent cells for transformation were prepared by a modification of the method of Sharma and Schimke (1996), where *E. coli* TOP10F' cells were grown in 2 x YT medium (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl). Growth of *E. coli* cells in a low salt medium was found to improve the efficiency of transformation. Electroporation was carried out at 1.7 V, 200 ohms, and 25 µFD, using electrocompetent cells and applying the method of Sharma and Schimke (1996). An aliquot (40µl) of competent cells was combined with 2 µl plasmid DNA,

mixed, and stored on ice for 1 min. The mixture was then transferred to a chilled 0.1-cm electroporation cuvette (Bio-Rad, Hercules, CA) and electroporation was carried out in a Bio-Rad Gene Pulser according to the manufacturer's instructions. A pulse with a time constant of 4-5 ms indicated that adequate transformation had occurred. The cuvette was immediately removed and 1 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added to revive cells (Sharma and Schimke, 1996). The mixture was transferred to sterile polypropylene tube and incubated with shaking for 1 h at 37°C. Cells were plated on LB-Zeocin plates with IPTG, for selection of transformants.

5.3.7 Southern Blotting

A Turboblotter, Rapid Downward Transfer Systems (Shleicher and Schuell, Keene, NH.) was used for preparation of Southern blots. The principle applied was the capillary transfer of DNA from an agarose gel onto a nylon membrane, using a high-salt transfer buffer to promote binding of the DNA to the membrane (Southern, 1975; Chomczynski, 1992). A 0.7% agarose gel in TAE (60 mM Tris/ 5 mM acetate/ 1 mM EDTA) buffer, pH 8.1, was run, loading the following samples for blotting: Digoxigenin labeled DNA molecular weight markers, *Lactobacillus amylovorus EcoR*I digest (20 µg) or *EcoR*I digested recombinant plasmids; digoxigenin labeled DNA probe as a positive control (50 pg); unlabeled DNA probe

as a control (500 pg, 250 pg, 50 pg). DNA was transferred to a positively charged nylon membrane using the neutral transfer buffer system, according to the manufacturer's instructions. The apparatus was able to transfer DNA without causing weight-induced gel flattening (Lichtenstein *et al.* 1990). DNA was fixed to the membrane by baking at 100°C for 30 min.

5.3.8 Replica plating and colony transfer for hybridization studies

In order to identify which recombinant clones contained plasmids carrying the glucoamylase gene, it was necessary to perform colony hybridization by first transferring colonies to nylon membrane discs. First, replica LB-Zeocin plates were prepared from master plates containing recombinant colonies, using replica plating apparatus of velveteen squares (6") and a replica block (Clontech Laboratories, Inc., Palo Alto, CA). Replica plating was carried out according to the manufacturer's instructions. Replica plates were marked and the corresponding areas on the nylon membrane disc were marked by making holes with a sterile needle, in order to later identify colonies. A procedure of denaturation, neutralization and fixing of DNA from colonies on nylon discs was followed, according to the manufacturer's instructions. DNA was fixed to the membrane by baking at 100°C for 30 min.

5.3.9 Preparation of the DNA probe

Glucoamylases from various organisms have been shown to contain four highly conserved regions in their amino acid sequences (Ohnishi *et al.* 1992). This fact was used in preparing a probe from the *STA2* gene of *Saccharomyces cerevisiae* var. *diastaticus* for gene cloning. A digoxigenin labeled probe was prepared thus eliminating the need to handle radioactive material, and providing an efficient means of hybridization. A digoxigenin (DIG) labeled probe for the glucoamylase gene was constructed using an Xho 1 digest of the pSA3 plasmid carrying the *S. diastaticus STA2* gene. DIG labeling was conducted using DIG-High Prime (Boehringer), a mixture of random primers; 1 U/µl Klenow enzyme; 1 mM each of dATP, dCTP, dGTP and 0.65 mM dTTP; 0.35 mM DIG-11-dUTP, alkali-labile, and 5 x stabilized reaction buffer in glycerol, 50% (v/v). The molar ratio of DIG-11-dUTP to dTTP was adjusted to ensure that every 20th-25th nucleotide in the newly synthesized DNA is modified with DIG. The procedure of DNA denaturation, labeling and DNA purification, was conducted according to the manufacturer's instructions. One µg of DNA was labeled for 1 h to obtain a minimum of 850 ng DIG-labeled DNA.

A more specific DNA probe containing the glucoamylase gene coding fragment *STA2* of pSA3, was prepared by removing the pBR322 sequences which form part of pSA3. This was achieved by performing a complete Sal1 digest of pSA3. The small fragment of less than 2 Kb (Fig. 5.2) was calculated to contain

the major part of the glucoamylase *STA2* gene coding region. The digest was run on a 1% low melting point (LMP) agarose gel in TAE (60 mM Tris/ 5 mM acetate/ 1 mM EDTA) buffer. The *STA2* gene coding fragment was extracted by first melting the gel at 65°C, then incubating the solution with ß-agarase at 40°C to breakdown the agarose matrix as outlined in the manufacturer's instructions. The DNA was extracted and purified, then labeled with digoxigenin, using DIG-High Prime.

5.3.10 Hybridization and selection of transformants

The southern blot of the genomic DNA digests was used to determine if the digoxigenin labeled pSA3 containing the *STA2* glucoamylase gene coding region, had any homology to a genomic DNA fragment. Hybridization was conducted in hybridization bottles at 65°C in an Autoblot Micro Hybridization Oven (Bellco Glass, Inc., Vineland, NJ). Pre-hybridization was conducted by immersing the membrane in hybridization buffer (5xSSC; blocking reagent, 1% w/v; N-lauroylsarcosine, 0.1% w/v; SDS, 0.02% w/v) for 2 h. Then, hybridization was conducted with the heat denatured DIG-labeled probe dissolved in hybridization buffer, for at least 6 h. For colony hybridization, the nylon discs were sealed in polypropylene pouches for pre-hybridization and hybridization at 65°C in order to carry out a large number of assays at the same time.

The nylon membranes were washed at a high stringency level, with a

solution of 0.1xSSC and SDS (0.1%, w/v) at 65°C according to the manufacturer's instructions. DIG-labeled DNA probe hybridized to target nucleic acids was detected by an enzyme-linked immunoassay using an antibody-conjugate, anti-digoxigenin alkaline phosphatase conjugate (anti-DIG-AP), supplied with the DIG-nucleic acid detection kit. This was followed by an enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) to produce an insoluble blue precipitate, which indicated hybrid molecules. After stringent washing with blocking reagent, color detection was left overnight to show up any areas of hybridization of the probe with *Lb. amylovorus* DNA.

Genomic DNA fragments which were homologous to the DIG labeled probe were visualized as dark blue and brown dots on nylon discs in the positions which correspond to colonies. The colonies were identified on replica plates and subcultured in 2 ml LB-Zeocin medium. Plasmid mini-preps were done for all possible positive colonies. Recombinant plasmids were extracted, digested with EcoRI and a Southern blot was prepared as described previously. The glucoamylase probe was used in hybridization studies to determine which colony was indeed positive for glucoamylase activity.

Positive clones were also verified by determining if the glucoamylase gene was expressed with the production of an active enzyme. Cultures of all possible positive clones were prepared in 5 ml LB-Zeocin. Cells were harvested by centrifugation 4,000 g at 5 min, then the pellet was resuspended in a microfuge tube, in 1 ml of 0.05 M sodium acetate buffer, pH 4.5. Cell extracts were prepared

at 4°C using lead-free glass beads (Glen Mills Inc., Clifton, New Jersey) to break cells and free cell contents, by shaking in a Cell disintegrator, Model MM2, (Brinkmann Instruments (Canada) Ltd., Rexdale, ON, Canada) for 15 min. The mixture was centrifuged at 10,000 g for 10 min. The supernatant was removed and centrifuged once more to remove beads and other cell debris. The cell extract was assayed for glucoamylase activity as outlined below.

5.3.11 Glucoamylase assay

Glucoamylase activity was determined spectrophotometrically by a modification of the method of McCleary *et al* (1991). Enzyme solution (100 µl) was added to 100 µl of *p*-nitrophenyl-ß-maltoside solution (Megazyme Pty. Ltd., N.S.W., Australia). The mixture was incubated at 40°C for 5 h and the reaction was terminated by the addition of 100 µl of 2% Trizma base (Sigma). Activity was calculated as follows: Activity (Units/ml) = $\blacktriangle A_{410}/t$ x a/b x 1/17.8 x Dilution Factor, where $\blacktriangle A_{410}$ = Absorbance (reaction) - Absorbance (blank), t = Incubation time in min, a = Final reaction volume, b = Volume of enzyme and 17.8 = $\varepsilon_m M$ *p*-nitrophenol in 2% Trizma base.

One unit of enzyme activity was defined as the amount of enzyme required to release one µmole of *p*-nitrophenol from the substrate per min under defined assay conditions. Specific activity was defined as the units of activity per mg protein.

5.3.12 Protein assay

Protein was determined spectrophotometrically by the Bicinchoninic Acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as a standard (Smith *et al.* 1985).

5.3.13 Glucoamylase from recombinant *Escherichia coli*

5.3.13.1 Effects of pH and temperature

The effect of pH on the activity of purified enzyme was measured in 0.05 M sodium acetate buffer (pH 3.0 to 8.0). Enzyme solution (100 µl) was incubated with 100 µl of *p*-nitrophenol-ß-maltoside (Megazyme) in the appropriate pH buffer, for 5 h at 40°C. The reaction was terminated by the addition of 100 µl 2% Trizma base and the yellow color of *p*-nitrophenol developed was measured spectrophotometrically at 410 nm. The assays were conducted in triplicate.

The effect of temperature on glucoamylase activity was measured in the range of 21°C-70°C. The substrate *p*-nitrophenol-ß-maltoside in 0.05 M sodium acetate buffer (pH 4.5) was equilibrated for 5 min at the test temperature before an equal volume of crude enzyme was added. The mixture was then incubated for 5 h at the test temperature and the reaction was terminated with the addition of an equal volume of 2% Trizma base. Enzyme activity was assayed by the

spectrophotometric measurement of the amount *p*-nitrophenol released.

5.3.13.2 Temperature stability

To estimate the thermal stability of glucoamylase, crude extract was incubated at 40°C, 50°C, 55°C, 60°C, 70°C, 80°C and activity was monitored at 10 min intervals up to 1 h, by assaying for residual glucoamylase activity.

5.3.13.3 Restriction Enzyme Mapping

Plasmids from the recombinant clones producing glucoamylase activity were prepared from 500 ml cell cultures, using the alkaline lysis method described previously. To determine the restriction map of the inserts of recombinant plasmids, complete digests were prepared with *BamH*I, *Eco*RI, *Eco*RV, *Hind*III and *Pst*I. Also *BamH*I fragments were digested with *Eco*RI, *Eco*RV, *Hind*III, *Pst*I or *Eco*RI digests with the other enzymes. Plasmid digests were run on 0.7% agarose gels in TAE buffer and the banding pattern was analyzed.

5.4 RESULTS

5.4.1 Cloning of *Lactobacillus amylovorus* glucoamylase gene

When EcoRI genomic DNA digest was hybridized with the pSA3 DIG-

labeled probe, three bands were visible after 16 h color development (Fig. 5.3). This demonstrated that there was some homology between *Lb. amylovorus* genomic DNA and the *STA2* glucoamylase gene-coding region from *Saccharomyces diastaticus.* Clones containing recombinant plasmids were transferred to nylon disc membranes and DNA was denatured and fixed to these membranes. Spots on the membrane discs indicated where possible recombinant plasmids encoding the glucoamylase gene hybridized with the DIG-labeled probe. A total of 129 blue/brown spots were obtained from 50 membranes of colony hybridization studies, accounting for 129 out of 3 x 10^5 transformants.

These transformants were then verified for the presence of the glucoamylase encoding gene. Cells were cultured in 2 ml LB-Zeocin medium and plasmid mini-preps were done. Then recombinant plasmids were digested with EcoRI and a Southern blot was prepared with subsequent hybridization using the glucoamylase probe. Clones were also verified by assaying for glucoamylase activity in crude cell extract. After three trials and purification of the clones, three designated G1, G13 and G35 were found to produce reproducible levels of glucoamylase (Table 5.1) thus representing 0.001% of the total number of transformants. The results of hybridization studies of some recombinants (EcoRI plasmid digests) are shown in Fig. 5.4. Bands are visible where the probe hybridized to homologous regions of the *Lb. amylovorus* genomic DNA. There was, however, some hybridization with the pZerO-1.1 cloning vector, indicating that the probe was not 100% specific for the glucoamylase gene.

When analyzed by agarose gel electrophoresis the transformants carried a plasmid larger than pZErO-1.1 cloning vector. Physical maps of plasmids pG1, pG13, and pG35 were generated by restriction enzyme analysis to allow future localization of the glucoamylase gene on the insert. In Fig. 5.5 it can be seen that the restriction map of the plasmid pG13 (glucoamylase gene fragment cloned) is different from that of the *Saccharomyces STA2* gene (Pretorius *et al.* 1991). While the insert size of plasmid pG13 was estimated to be about 5.2 Kb, plasmid pG1 and pG35 were estimated at 6.4 Kb and 5.9 Kb, respectively. No cleavage sites were found for the enzyme *Hind*III. The enzymes *BamH*I, *Eco*RV and *Pst*I had one cleavage site each, while *EcoR*I had multiple cleavage sites in the insert. The inserts of plasmids pG35 and pG1 showed an overlap of restriction enzyme mapping sites and common bands were obtained in mapping experiments.

5.4.2 Characterization of the glucoamylase activity

The characteristics of the recombinant glucoamylase were compared with those of the wild type strain, *Lactobacillus amylovorus*. Both enzymes showed a similar pH range of 2.0-8.5 in sodium acetate buffer with the optimum pH at 6.0 (Fig. 5.6), however, the pH profiles of the recombinants were broader than that of the wild type. The effect of temperature on the enzyme activity is shown in Fig 5.7. The maximum activity was obtained between 40-45°C, and both recombinant and wild type enzymes retained about 20% activity at 4°C.

To estimate the thermal stability of the recombinant glucoamylase, the enzyme was incubated for 1 h at different temperatures. Samples were withdrawn periodically and residual activity was measured at 40°C. Both the recombinant and wild type enzymes were stable up to 50°C for over 1 h, and they were found to lose activity more rapidly at 60°C and over (Fig. 5.8).

5.5 DISCUSSION

Glucoamylase genes from many fungal strains have been cloned in *S. cerevisiae* to investigate simultaneous saccharification and fermentation as a means of simplifying the brewing process, as well as reducing overall production costs. Most glucoamylase genes which have been cloned, produce thermostable enzymes which are more beneficial for the conversion of starch to glucose and high fructose corn syrup (Pretorius *et al.* 1991). The production of a thermolabile glucoamylase would be useful in the brewing of low calorie beer, since the enzyme could be inactivated during the final pasteurization step.

The glucoamylase gene of *Lactobacillus amylovorus* has been successfully cloned in *Escherichia coli* TOP10F' cells. The expression levels of the three recombinant strains were very low when compared with the wild type but glucoamylase activity was nine times greater than the basal level of *E. coli* cells (Table 5.1). It was not possible to do a control experiment for plasmid effect in *E. coli* cells since p-Zer0-1.1 plasmids without inserts carry the *ccd*B gene, which

would be expressed, killing the cells. Low glucoamylase activity could be attributed to weak transcription from some cryptic promoter located upstream of the gene coding region in the recombinant plasmid. The *E. coli* cell may not have an adequate system to transcribe this foreign DNA. Eratt and Nasim (1989) also reported very low levels of glucoamylase activity in recombinant *S. cerevisiae* when they cloned the *S. diastaticus*, glucoamylase gene.

The pH of malt in brewing is about 6.0, and mashing enzymes operate within the range of 5.2-5.7, making it necessary to acidify the mash during this stage of the brewing process (Broderick, 1977). It should also be noted that wort used for fermentation has a pH range of 5.0-6.0 (Broderick, 1977). The glucoamylase produced by *Lb. amylovorus* is well suited for application at these pH ranges since maximum enzyme activity was obtained at pH 6.0. The pH optimum of the recombinant glucoamylase was also 6.0, which strongly supports the fact that both wild type and recombinant proteins are the same. The optimum temperature of both the wild type and clones appeared to be similar (40° - 45°C). Thus a thermolabile glucoamylase was cloned. Also from thermostability studies the enzymes of both wild type and clones could be said to be thermolabile above 55°C. This recombinant enzyme could therefore be inactivated by pasteurization temperatures (60°-65°C for 5 min) applied during the last stage of brewing.

To confirm the expression of the glucoamylase gene in TOP10F' cells, subcloning by unidirectional deletions, would have to be performed using the knowledge of the restriction map. A plasmid which carries the smallest insert, yet

still gives glucoamylase activity would be important for determining the nucleic acid sequence of the gene, as well as improving the gene expression. Further work must be done to find a strong promoter which would increase glucoamylase production in recombinant cells to the level of that in *Lb. amylovorus*. Then, ligating the glucoamylase gene in an *E. coli*-yeast shuttle vector like YEp13, brewer's yeast cells could be transformed. Glucoamylase gene expression and the potential application of this recombinant yeast cell in the brewing industry can then be investigated.

5.6 CONCLUSION

A glucoamylase encoding gene was cloned from a DNA library of *Lb. amylovorus*. The cloned DNA fragment contains at least a structural gene for the intracellular glucoamylase because *E. coli* TOP10F' cells transformed with a plasmid carrying this DNA segment produced glucoamylase having similar enzymatic characteristics (optimal pH and temperature) as those of the *Lb. amylovorus* glucoamylase. Cloning of this gene in *Saccharomyces cerevisiae* may lead to the development of novel, concerted processes with simultaneous saccharification and fermentation, especially in the brewing of beer where a thermolabile enzyme is required.

Table 5.1. Glucoamylase activity in transformed *Escherichia coli* cells carrying the *Lactobacillus amylovorus* glucoamylase gene sequence in pZer0-1.1 plasmid vectors.

Strain	Activity (10^{-2} Units/ml)	Specific Activity (10^{-3} Units/mg)
Clone G1	0.0042	0.0028
Clone G13	0.0026	0.0031
Clone G35	0.0024	0.0018
TOP10F' cells (control)	0.0003	0.0002
Lb. amylovorus (wild type)	4.5511	6.7112

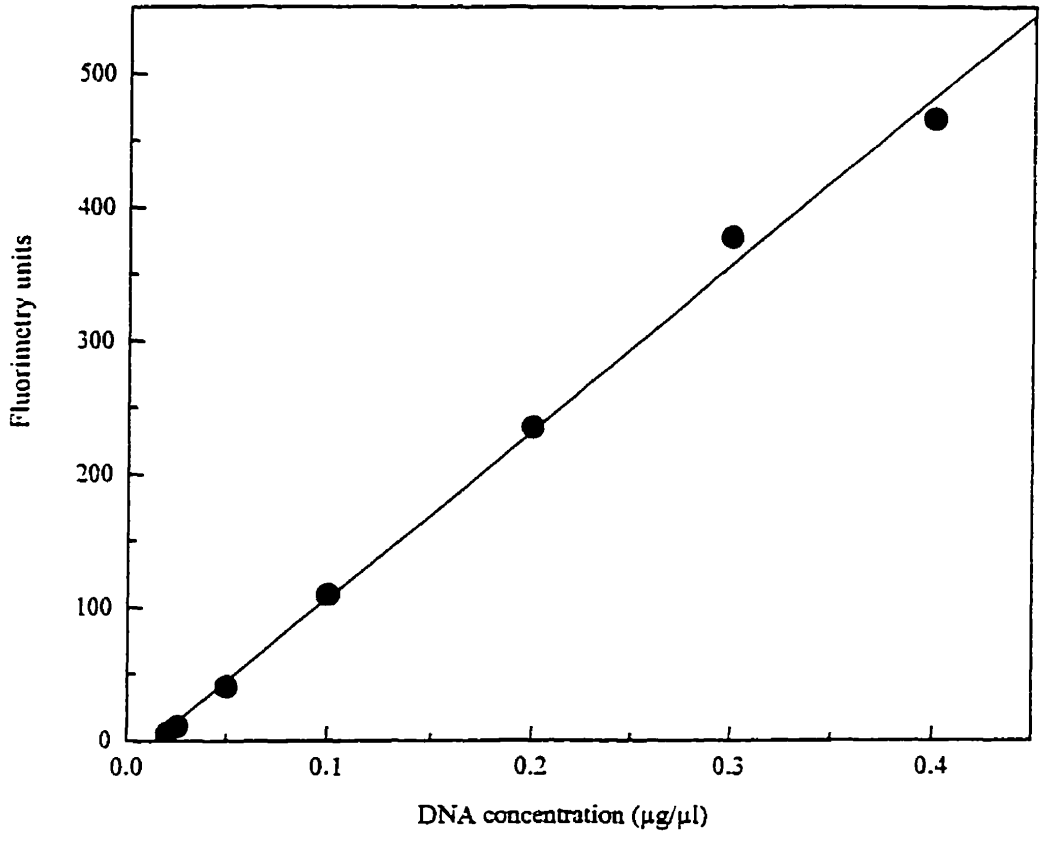


Figure 5.1. Calibration curve of pure calf thymus DNA in 10 µl capillary tubes using a TKO 120 fluorometer. R^2 = 0.9965, Y = -17.0833 + 1246.1946.

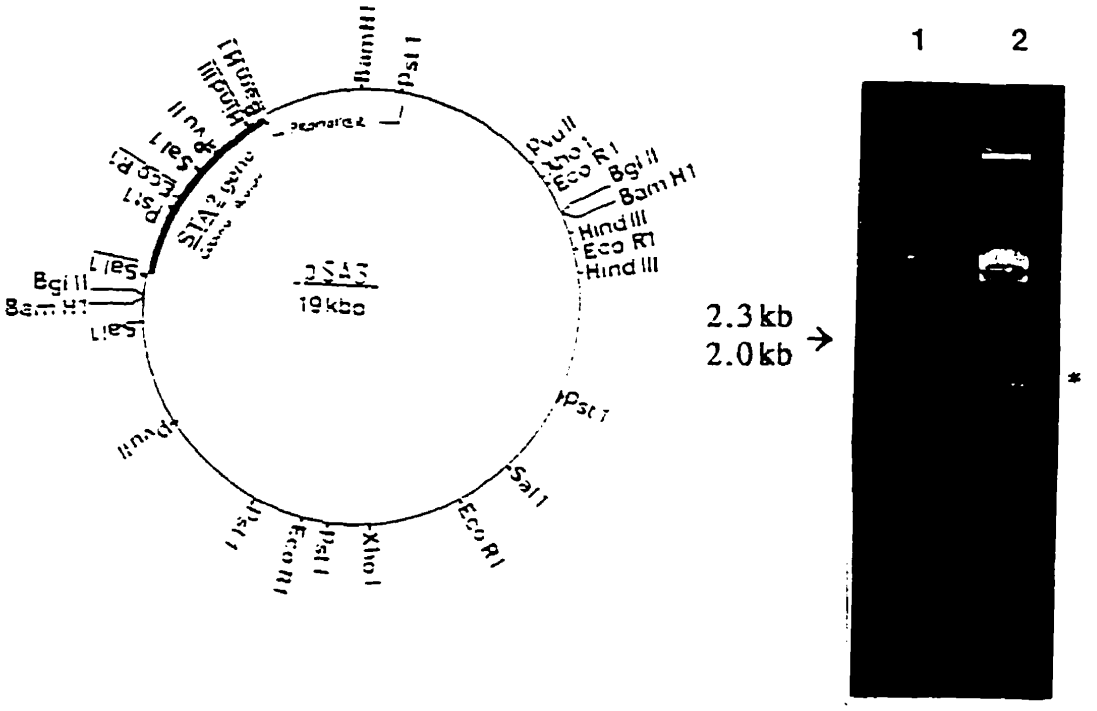


Figure 5.2. The pSA3 plasmid vector used for DNA probe preparation and the *Sal*1 digest formed. Lane 1, Molecular mass marker; Lane 2, *Sal*1 digest; (*) DNA band containing the *STA2* gene coding sequences.

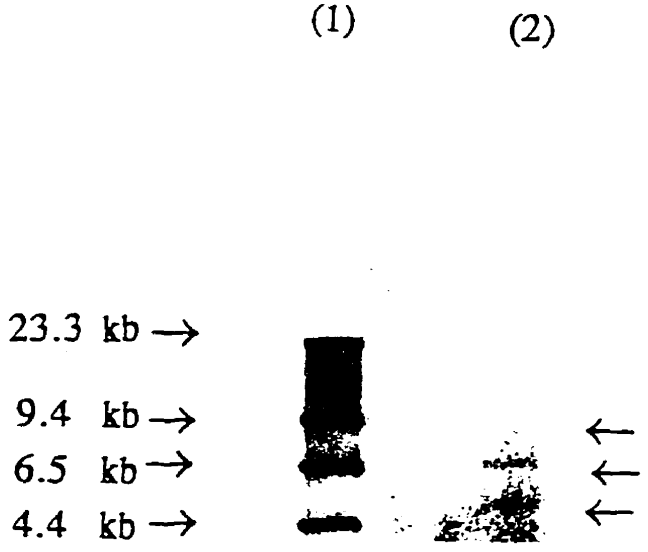


Figure 5.3. The DIG-labeled pSA3 DNA probe hybridized to genomic DNA from *Lactobacillus amylovorus*. Lane 1, Molecular weight markers; Lane 2, *EcoR*I digestion of genomic DNA. Three DNA bands hybridized to the DIG-labeled probe.

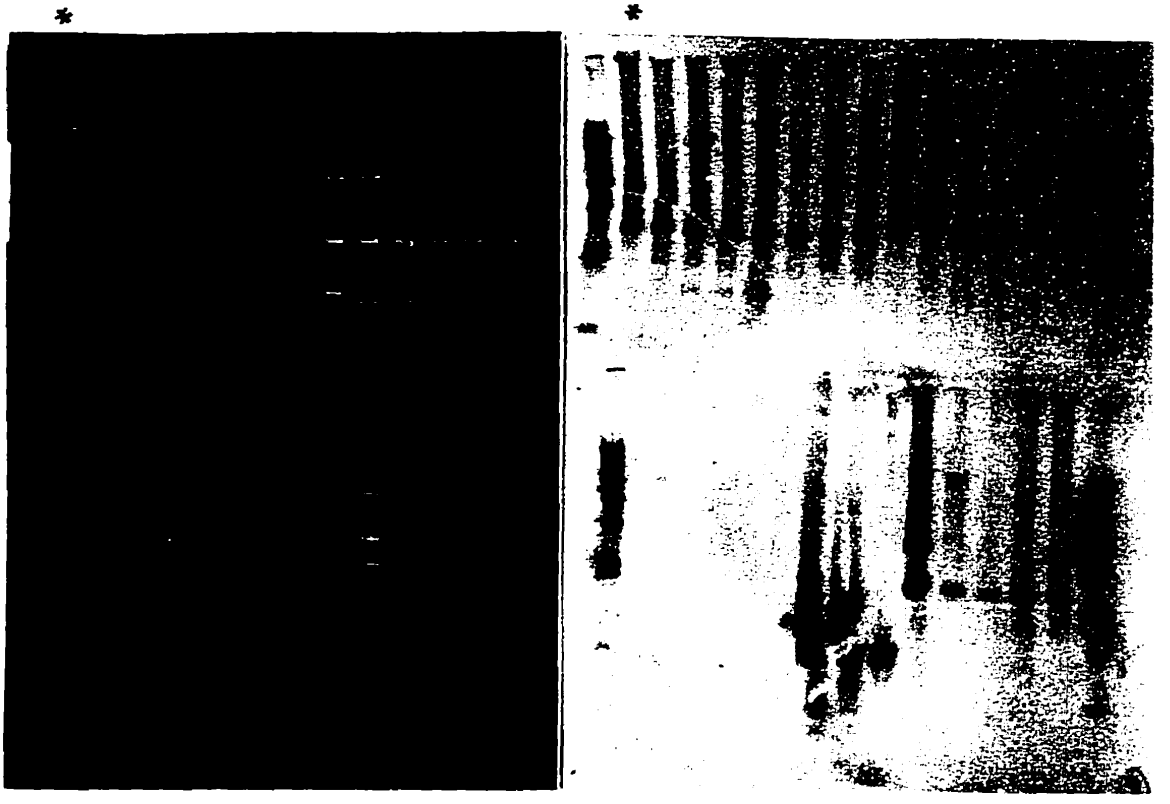


Figure 5.4. (A) *EcoR*I digests of recombinant plasmids from positive colonies containing the glucoamylase gene. (B) The corresponding Southern blot of *EcoR*I plasmid digests hybridized with the digoxigenin labeled, *STA2* DNA probe. Lane of pG1 *EcoR*I digest (*). Lane of pG13 EcoR1 digest (#).

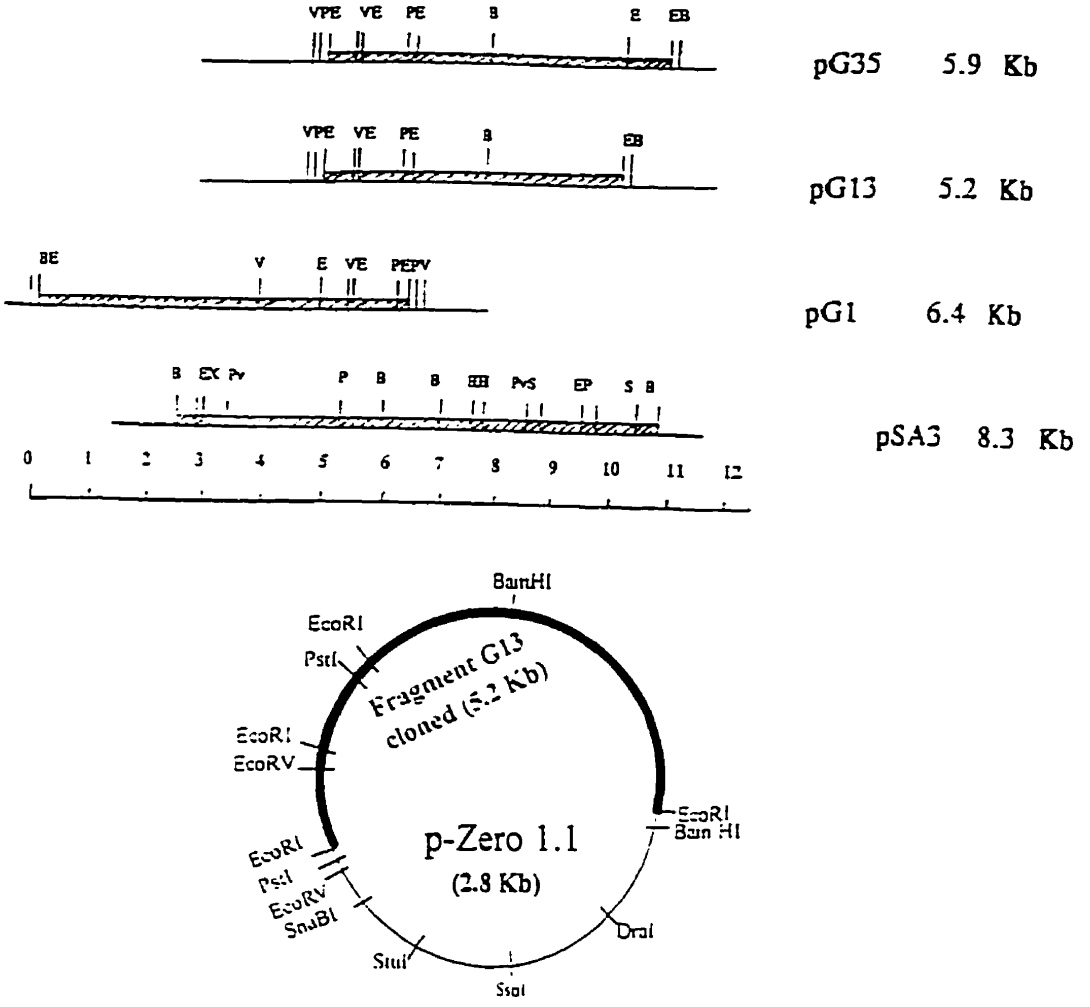


Figure 5.5. Physical maps of DNA inserts carrying the glucoamylase gene of *Lactobacillus amylovorus*. These maps of pG1, pG13 and pG35 are compared with that of the *STA2* glucoamylase gene from *Saccharomyces diastaticus*. B, *BamH*I; E, *EcoR*I; V, *EcoRV*; H, *Hind*III; Pv, *Pvu*II; P, *Pst*I; S, *Sal*I. The circle represents the restriction enzyme map of pG13, showing the p-ZerO-1.1 vector used for cloning.

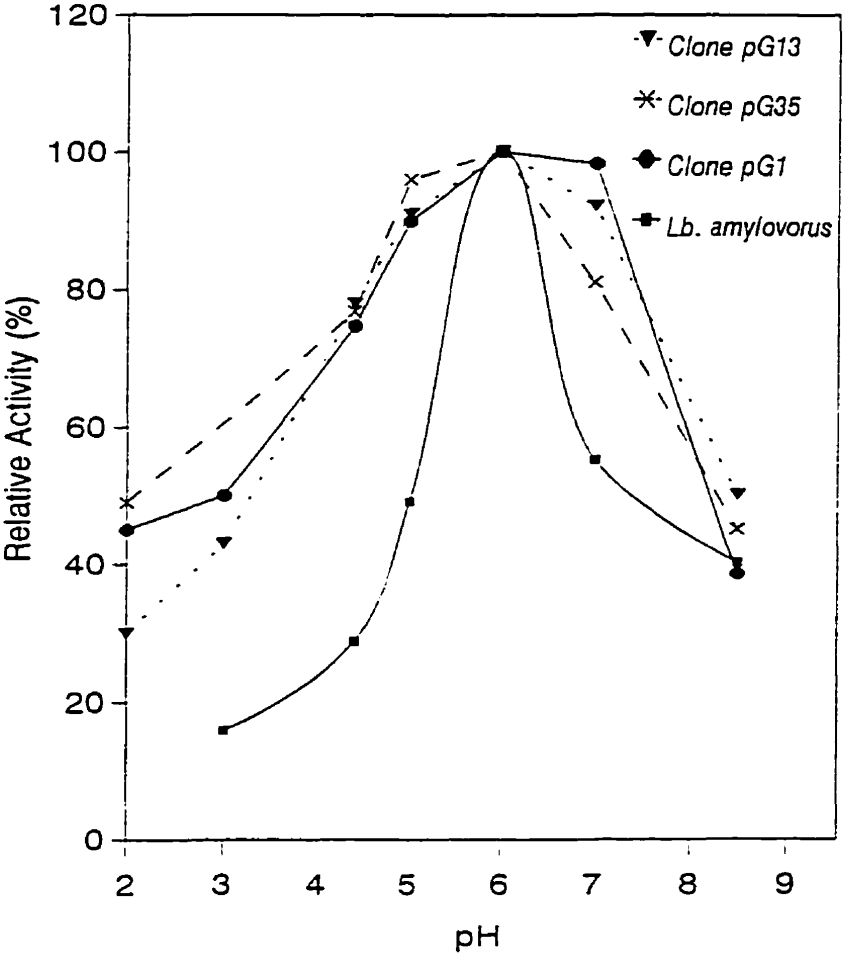


Figure 5.6. Effect of pH on recombinant glucoamylase activity of clones G13, G35 and G1, compared with that of wild type *Lactobacillus amylovorus*.

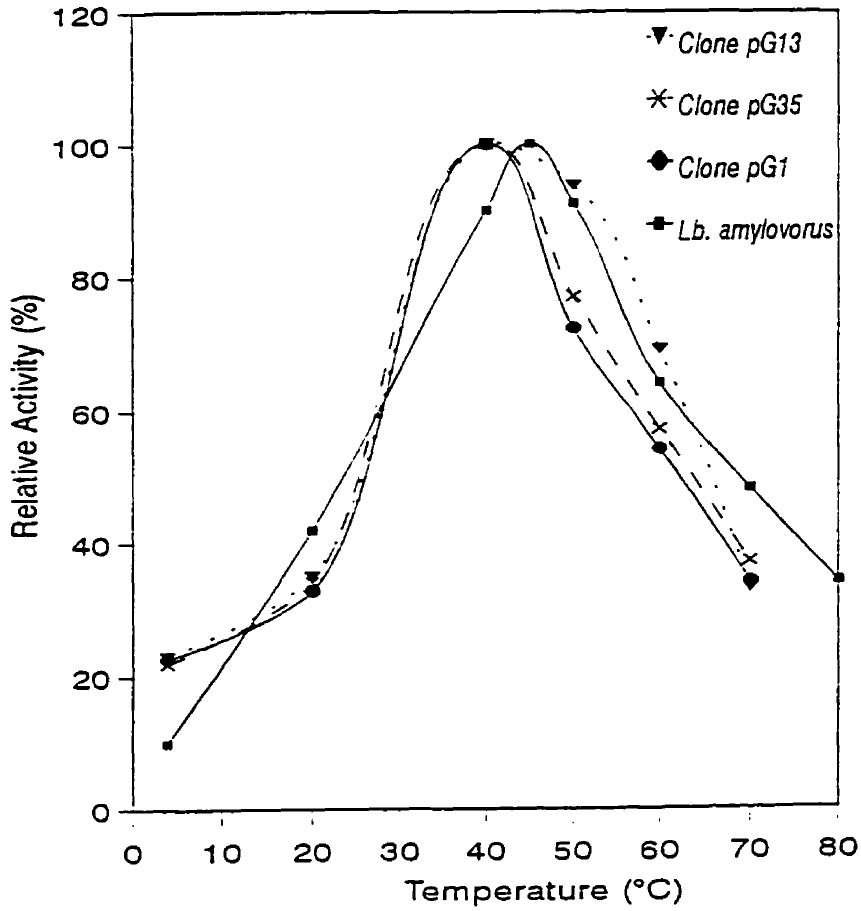


Figure 5.7: Effect of temperature on recombinant glucoamylase activity of clones G13, G35 and G1, compared with that of wild type *Lactobacillus amylovorus*.

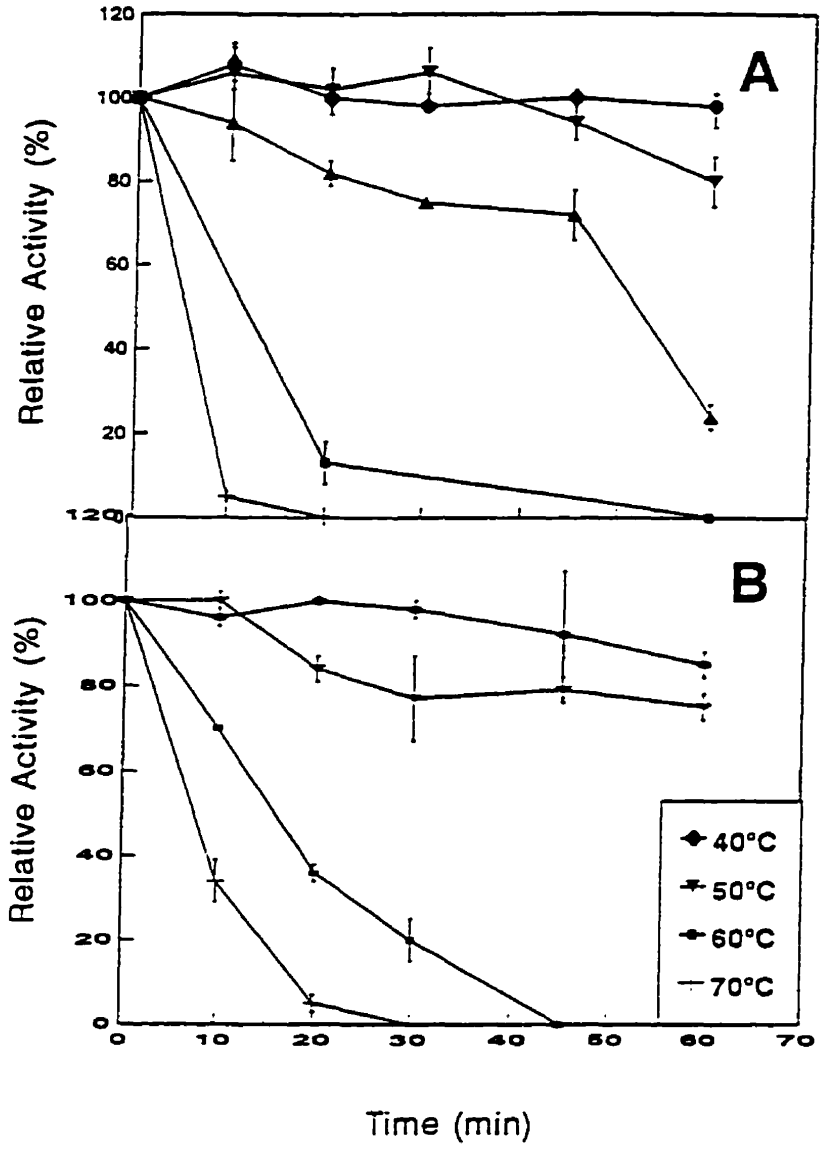


Figure 5.8. Thermostability of glucoamylase from *Lactobacillus amylovorus* (A) and recombinant *Escherichia coli* (B). The crude extracts were incubated at 40°C, 50°C, 60°C, 70°C for the times indicated and then assayed for residual glucoamylase activity at 40°C.

GENERAL CONCLUSIONS

Glucoamylase was produced intracellularly in *Lactobacillus amylovorus* induced by dextrin in the growth medium. In order to investigate the potential of glucoamylase for application in the food industry, it was necessary to maximize glucoamylase production, and purify and characterize the enzyme. Maximal growth and glucoamylase production in *Lactobacillus amylovorus* ATCC 33621 were obtained after 16-18 h of growth when cells were cultivated in MRS medium supplemented with 1% (w/v) dextrin, at 37°C and pH 5.5.

The results of purification, electrophoretic analysis and activity staining revealed a monomeric enzyme of about 47 kD. The optimal pH of glucoamylase from *Lb. amylovorus* was 6.0 and thus the enzyme can be applied in the brewing of low calorie beer, where the pH of malt is about 6.0, and mashing enzymes operate within the range of 5.2-5.7. The crude enzyme had optimal activity at 55°C, which is relatively thermolabile in contrast to other bacterial glucoamylases which have optimal activities of 65°-75°C. The temperature stability profile, however, indicated that glucoamylase was not completely inactivated after 10 min incubation at 60°C. But, a potential for application in brewing was identified, since the stages of boiling of wort with hops, and final pasteurization at 60°C-65°C, would be adequate to inactivate glucoamylase activity.

Glucoamylase from *Lb. amylovorus* reacted preferentially with polysaccharides as compared to short chain oligosaccharides. Thus, glucoamylase

activity was proportional to the length of the oligosaccharide chain. This observation was further supported by the fact that there was a decrease in K_m with longer substrate chain length. The low K_m for dextrin would be beneficial in the hydrolysis of dextrins from malted barley. Glucoamylase was sensitive to glucose, the main end product of the enzyme reaction, at 10 mM. This factor would be detrimental in brewing applications if free glucose were allowed to accumulate without being fermented by brewer's yeast.

There was no inhibition of enzyme activity in the presence of EDTA and 1,10-phenanthroline. This indicated that divalent cations may not be involved in the catalytic mechanism of glucoamylase. However, glucoamylase from *Lb. amylovorus* was inhibited by heavy metals like Pb^{2+} and this behavior is typical of other characterized enzymes. The presence of EDTA was also found to enhance enzyme activity. Glucoamylase was inhibited 22% by p-chloromecuribenzoic acid at 10 mM. Inhibition by other sulfhydryl inhibitors like iodoacetate, suggested the presence of a sulfhydryl group near the enzyme active site, and its possible involvement in catalysis.

A glucoamylase producing gene was cloned from a DNA library of *Lb. amylovorus*. This cloned DNA fragment contained a structural gene for the intracellular, thermolabile glucoamylase since *E. coli* TOP10F' cells transformed with a plasmid carrying this DNA segment produced glucoamylase. This enzyme had similar enzymatic characteristics (optimal pH and temperature) as those of the *Lb. amylovorus* purified glucoamylase. The enzymes of both wild type and clones

were rapidly inactivated at temperatures above 55°C.

From the results of this investigation one can conclude that glucoamylase from *Lactobacillus amylovorus* showed a good potential for application in the brewing of low calorie beer. The costly process of isolating an intracellular enzyme does not make it competitive with commercially prepared mold glucoamylases. However, the cloning of the glucoamylase gene in *Saccharomyces cerevisiae* would provide an efficient means of exploiting the properties of this enzyme. The techniques of protein engineering could be used to modify this glucoamylase to improve its thermolabile properties. Also, further investigation must be done to improve the expression of the glucoamylase gene and its cloning in *Saccharomyces cerevisiae*. New processes with simultaneous saccharification and fermentation may then be possible.

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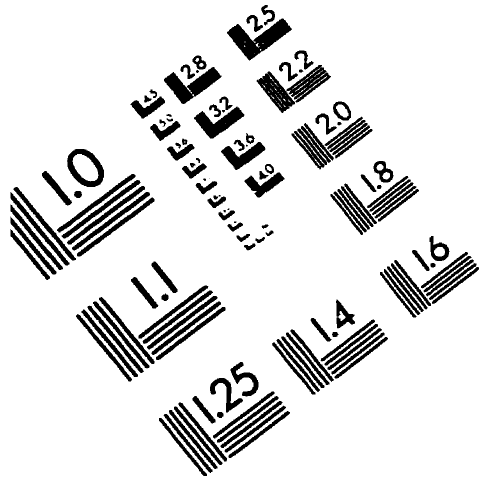
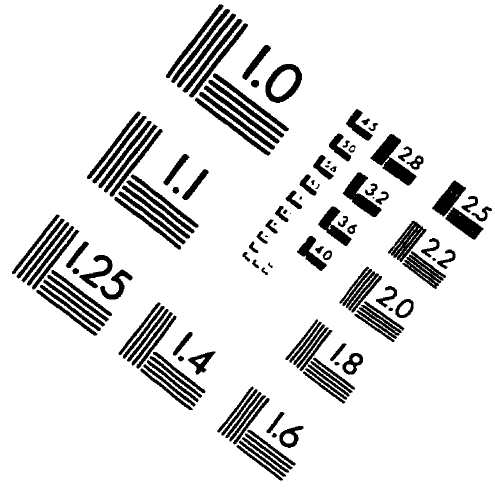
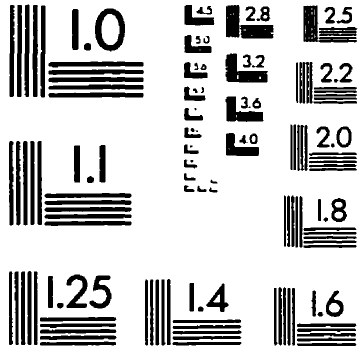
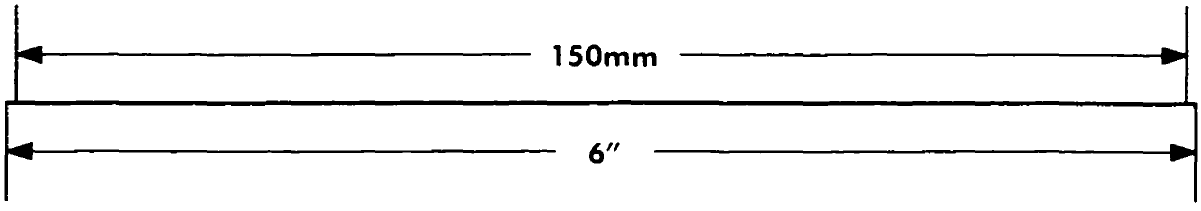
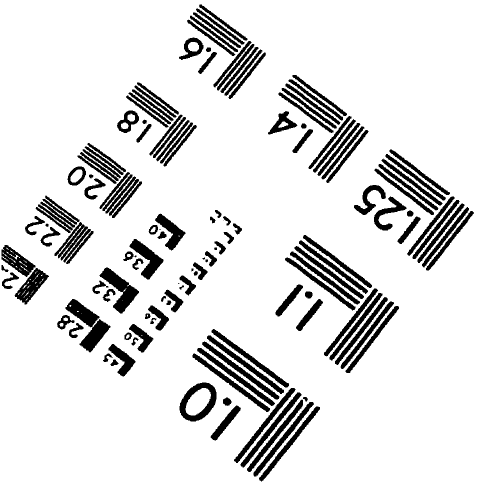
IMAGE EVALUATION TEST TARGET (QA-3)

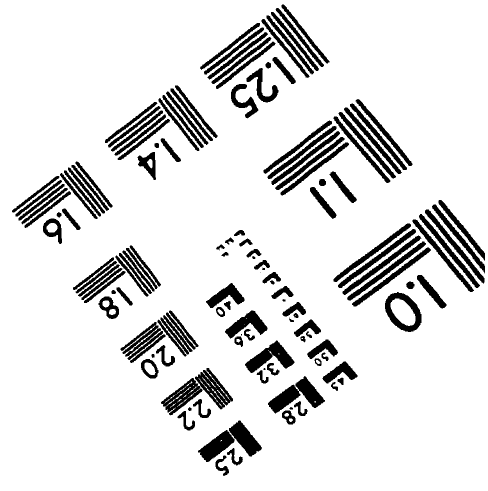












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