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BIOSYNTHESIS OF CELLULASE-SYSTEM FROM *TRICHODERMA RESEEI* AND ITS CHARACTERISTICS

A DISSERTATION BY

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Submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Suggested short title:

CELLULASE PRODUCTION, CELLULOSE HYDROLYSIS AND ETHANOL PRODUCTION

DEDICATED TO

My late mother, Bernadette Akatariba Awafo who is never far away.

FOREWORD

This thesis is presented in the form of original papers meant for publication in journals. The first two chapters comprise the general introduction with stated goals of the research and a literature survey of the subject matter. The next six chapters are complete manuscripts suitable for publication. Each of these six chapters opens with a connecting statement that links them altogether as an integrated approach for ethanol production from waste materials. The dissertation concludes with summary of the major results of the study.
This format has been approved by the Faculty of Graduate Studies and Research, McGill University in accordance with the conditions outlined in the Guidelines for Thesis Preparation, Thesis Specification, section 3 entitled "Traditional and manuscript-based thesis" which are as follows:

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

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The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography of the reference list.

Additional material must be provided where appropriate (e.g., in appendices) and in sufficient detail to allow clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
In the case of manuscripts co-authored by the candiadte and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all authors of the co-authored papers. Under no circumstances can a co-author of any component of such thesis serve as an examiner of that thesis.

This dissertation is the responsibility of the candidate but was co-supervised by Dr. D.S. Chahal, Centre de Recherche en Microbiologie Appliquée, Institut Armand-Frappier, Université du Quebec and Dr. B.K. Simpson, Department of Food Science and Agricultural Chemistry, Macdonald Campus, McGill University.

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Awafo, V.A., Chahal, D.S., and Simpson, B.K. 1997. Comparative evaluation of mild sodium hydroxide and its combination with steam explosion as pretreatments of wheat straw for the production of cellulase-systems of two *T. reesei* mutants under solid-state fermentation conditions. Bioresource Biotechnology.

Awafo, V.A., Chahal, D.S., and Simpson, B.K. 1997. Evaluation of a novel pan-bioreactor for cellulase-system biosynthesis under solid-state fermentation conditions. Biotechnol. Bioeng.

Awafo, V.A., Chahal, D.S., and Simpson, B.K. 1997. Production of ethanol through enzymatic hydrolysis of delignified wheat straw. J. Food Biochemistry

Awafo, V.A., Chahal, D.S., and Simpson, B.K. 1997. Optimization of ethanol production by *Sacchromyces cerevisiae* and *Pichia stipitis*: A response surface model for wheat straw concentration and cellulase-system loading for simultaneous hydrolysis and fermentation. Appl. Biochem. Biotechnology

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Awafo, V.A., Chahal, D.S., and Simpson, B.K. 1996. Response surface optimization of steam explosion for cellulase production by *T. reesei* QMY-1. 96th General meeting of the American Society for Microbiology, May 19-23, New Orleans, Louisiana, USA

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ABSTRACT

There are generally four factors recognized as delimiting in the study of lignocelluloses for fuel ethanol production, viz., the source of the cellulase-system and its quality characteristics for cellulose hydrolysis, the substrate and pretreatment method, the process for cellulase production and bioreactor design, and the ability of yeast to ferment mixed hexose and pentose sugars. Wheat straw (WS) and *T. reesei* mutants were used in the study to evaluate the production of cellulase-systems. Hydrolysis of cellulose revealed the superiority of mild NaOH pretreatment over steam explosion for cellulase production with *T. reesei* MCG 80 and QMY-1. Response surface models were capable of predicting that NaOH could be used for the pretreatment of WS at 4% (w/w) without urea in the fermentation medium to yield optimum filter paper activity (FPA) of 9.9 IU/mL (247 IU/g WS) and beta-glucosidase activity (ßGA) of 6.4 IU/mL (159 IU/g WS) under solid-state fermentation (SSF) conditions. Multiple regression analysis with multiple coefficients of correlation, R, between 0.957 and 0.99 from the experimental data showed close agreement between the cellulase activities (FPA and ßGA) from the experiments and predicted values.

The superiority of SSF over liquid-state fermentation (LSF) in the production of cellulase-systems was also established, and a prototype pan-bioreactor showed good potential for upgrading cellulase production under SSF conditions. The economics of fuel ethanol production was considered in the optimization model that sought to establish threshold cellulase loadings needed to achieve maximum cellulose hydrolysis for fermentation. High substrate concentrations of up to 7.5% were hydrolyzed with cellulase

loadings of 24 -30 IU/g and fermented by *Pichia stipitis* to achieve 90-100% conversion into ethanol.

Crude unextracted cellulase yielded over 90% hydrolysis of delignified wheat straw and proved to be better than extracted cellulase and commercial cellulases for the hydrolysis of pure cellulose and pretreated wheat straw. Studies were also conducted to demonstrate the importance of the ratio of ßGA- to FPA in cellulose hydrolysis which showed that ratios closer to one (1), produced more sugars and lowered the cellobiose content in the hydrolysates. It was also shown that the source of the cellulase is important in eliminating the accumulation of cellobiose during hydrolysis as was demonstrated with cellulase from mixed cultures of *T. reesei* and *Aspergillus phoenicis*. Higher ßGA from the latter were implicated since *A. phoenicis* is a good ß-glucosidase producer.

Delignified wheat straw at 5% concentration when subjected to separate hydrolysis and fermentation and simultaneous hydrolysis and fermentation resulted in similar volumetric productivities (g/L/h) of ethanol.

RESUMÉ

En général, il existe quatre facteurs déterminants dans l'étude des lignocelluloses pour la production de l'éthanol combustible. Ces facteurs sont les suivants: la source du système cellulasique et ses qualités caractéristiques pour l'hydrolyse de la cellulose, le substrat et la méthode de prétraitement, le procédé de production de la cellulase et la conception ainsi que l'habilité de la levure à fermenter un mélange de sucre pentose et hexose. Dans la présente étude, l'hydrolyse de la cellulose par la cellulase issue de *T. reesei* MCG-80 et QMY-1 a révélé qu'un prétraitement au NaOH donnait un résultat supérieur à celui fait à l'explosion à vapeur . À l'aide de la méthologie des models de réponse-surface il a été possible de prédire qu'en se servant d'un milieu de fermentation sans urée contenant de la paille de blé prétraitée au NaOH à 4% (p/p) on produirait une activité optimale de papier-filtre de 9,9 UI/mL (247 UI/g de paille de blé) et une activité de la bêta-glucosidase de 6.4 UI/mL (159 UI/g de paille de blé) dans des conditions de fermentation à l'état solide. Des analyses de regression multiples avec des coefficents de corrélation R compris entre 0,957 et 0,99 des données expérimentales ont montré que les valeurs de l activité du papier-filtre et celles de la bêta-glucosidase obtenues expérimentalement étaient proches de celles prédites théoriquement. L'efficacité de la fermentation à l'état solide par rapport à la fermentation à l'état liquide pour la production de cellulase a été vérifiée et un prototype de bioréacteur a indiqué le potentiel croissant de la production de cellulases dans les conditions de fermentations à l'état solide. Le côté économique de la production de l'éthanol combustible a été pris en compte dans l'optimisation d'un model de

bioréacteur pour hydrolyser une quantité maximale de cellulose au cours de la fermentation. Une forte concentration de substrat allant jusqu à 7,5% a été hydrolysé avec une concentration de cellulase de 24 à 30 UI/g puis fermenté par *Pichia stipitis* permettant d'obtenir 90 à 100% d éthanol. La cellulase brute non extraite a permis d'hydrolyser plus de 90% de paille de ble délignifiée. Ceci prouve que la cellulase non extraite est plus efficace pour l'hydrolyse de la cellulose pure et de la paille de blé prétraitée par rapport à celle extraite ou commercialisée. Des études ont été aussi menées pour démontrer l'importance du rapport entre la bêta glucosidase et l'activité du papier-filtre au cours de l'hydrolyse de la cellulose. La valeur du rapport tendait vers un, montrant qu'il se produisait plus de sucre diminuant ainsi la quantité de cellobiose dans les hydrolysats. Il a été prouvé que la source de cellulase était importante dans l'élimination de la cellobiose au cours de l'hydrolyse en se servant de cultures mixtes de *T. reesei* et d'*Aspergillus phoenicis* . On peut dire qu'une forte concentration de la bêta-glucosidase a joué un important rôle dans cette réaction, sachant qu'*Aspergillus phoenicis* est un bon producteur de cette enzyme. Une expérience au cours de laquelle 5% de paille de blé delignifiée a été soumise séparement puis simultanement à l'hydrolyse et à la fermentation a permis d'obtenir la même quantité d'éthanol en g/L/h dans chacun des deux cas.

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CONTRIBUTION TO KNOWLEDGE

1. Comparative assessment of solid-state fermentation (SSF) and liquid state fermentation (LSF) of wheat straw for cellulase production to establish the superiority of SSF over LSF in cellulose hydrolysis by relating their ß-glucosidase activity (ßGA) to Filter paper activity (FPA) ratios.

2. A novel bioreactor system was assessed to relate its nature of design to cellulase-system production and cellulose hydrolysis. Information from the novel pan-bioreactor has potential for scaling up cellulase-system production.

3. The first comprehensive report that uses optimization models to assess pretreatment types and levels for cellulase-system production, cellulose hydrolysis and ethanol production all with the same starting lignocellulosic substrate. Optimum levels of these parameters were established.

4. Unextracted cellulase-system from *T. reesei* outperformed extracted cellulase systems and commercial cellulases in cellulose hydrolysis. No such study in solid-state fermentation systems is reported. The basis of extraction of cellulase-systems might render the cellulase activity values as artifacts of the dilution process if contribution of unutilized cellulose in the unextracted cellulase is minimal.

5. The first definitive illustration that cellobiose accumulation could be eliminated or minimized considerably during cellulose hydrolysis from good quality cellulase-systems obtained from mixed or single culture systems under solid-state fermentation conditions.

6. Studies of the adaptation of various yeast to ferment lignocellulosic substrates with either processes of separate hydrolysis and fermentation or simultaneous hydrolysis and fermentation under optimized conditions were capable of declaring the best fermenting yeast for mixed hexose and pentose sugars.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
AP	*Aspergillus phoenicis*
ARS	Agricultural Research Service
ATCC	American Type Culture Collection
ßGA	Beta glucosidase activity
CBH	Cellobiohydrolase
CEC	Crude Extracted Cellulase-system
CEC-H	Crude Extracted Cellulase-system Hydrolysate
CMC	Carboxymethyl Cellulose
CUC	Crude Unextracted Cellulase-system
CUC-H	Crude Unextracted Cellulase-system Hydrolysate
DF	Degrees of Freedom
DWS	Delignified Wheat Straw
E.C	Enzyme Commission
EG	Endoglucanase
FPA	Filter Paper Activity
FPU	Filter Paper Units
G-H	Genecor hydrolysate
HF	Separate Hydrolysis and Fermentation
HFCS	High Fructose Corn Syrup
HPLC	High Performance Liquid Chromatography
I-H	Iogen hydrolysate
IUPAC	International Union of Pure and Applied Chemists
LSF	Liquid-State fermentation
MBP	Microbial Biomass Protein
MS	Mean square
NRRL	Northern Regional Research Center
NWS	Sodium hydroxide pretreated wheat straw
PEG	Polyethylene glycol
R	Multiple coefficient of correlation
R^2	Multiple coefficient of determination
SAXS	Small angle X-ray scattering
SCP	Single Cell Protein
SHF	Simultaneous Hydrolysis and Fermentation
SS	Sum of squares
SSF	Solid-State Fermentation
SWS	Steam pretreated Wheat Straw
USDA	United States Department of Agriculture
WS	Wheat straw

CHAPTER 1

INTRODUCTION

Glucose and xylose are the two major sugars that can be obtained by the hydrolysis of lignocellulosic biomass as important feedstocks for production of foods, pharmaceuticals, and various chemicals. Traditionally, glucose is derived from cane sugars, cereals, grain crops, and root tubers, and then transformed into end products such as ethanol and high fructose corn syrup (HFCS). However, glucose can also be obtained from lignocelluloses thus removing equivalent amounts of the traditional glucose sources and making them more available for human consumption (Biomass Panel, 1983).

Lignocelluloses derived from agricultural residues, forest wastes, and by-products from agricultural related industries, are all potential substrates that can be converted into useful products by enzymatic hydrolysis to simple sugars. Research into the potential applications of lignocelluloses (lignocellulosic materials) can generally be classified into three phases. Phase I, i.e., the discovery phase, was heralded by the accidental discovery of cellulases as a result of their destructive properties on war time materials during the second world war. The cellulases are a mixture of hydrolytic enzymes, herein referred to as the cellulase-system, that interactively and cooperatively attack and saccharify cellulose to release glucose units. There was not much excitement during phase I and only a few laboratories notably the US Army Natick Development Center, Natick, Massachusetts were involved. Phase II, the more exciting or bench-top phase, started in

the early seventies, when the world experienced the first oil crisis. It was only then acknowledged that oil resources are limited and their access uncertain. To date, it is certain that oil prices will continue to rise because the resources continue to be more and more difficult to get at in the deep sea or in the middle of nowhere. This situation even gets aggravated as the socio-political climate in the major oil producing countries in the Middle East gets more and more uncertain by the day, luring countries to go to war over oil resources. In a horizontal plane, during this period, lignocelluloses became the potential panacea to the world's food/feed problems first as single cell protein (SCP) and later as microbial biomass protein (MBP). The excitement peaked off as no simple commercial solutions with lignocelluloses were eminent. It has been over two decades now and the best that has come out of seeking alternatives to oil as transportation fuel is a 10 % fuel ethanol-gas blend, gasohol, derived mostly from cane sugar and cereal crops.

Phase III, the latent phase (which is now), is characterized by more careful assessments of the individual process parameters for improvements and economics of production of fuel ethanol. It is still, however, clear that the potential of lignocelluloses to provide a solution to the ever dwindling oil supplies and as enzyme sources to aid several food, animal feed, and pharmaceutical processes is quite enormous provided this universally abundant and largely underutilized renewable resource can be efficiently and economically harnessed for enzyme production and for hydrolysis into fermentable sugars.

Figure 1-1 shows a typical route for fuel ethanol production from lignocelluloses. The hydrolysis step is overburdened with problems arising from the nature of the lignocelluloses. It is heterogeneous and recalcitrant to either acid or enzymatic hydrolysis

and, therefore, requires some form of pretreatment. Attention is paid more to enzymatic hydrolysis than to acid hydrolysis because the former is more environmentally friendly, requires less capital investment, recovers product with more uniform physico-chemical properties, and has higher yields and turnover rates. However, the production of the enzyme - system that hydrolyzes lignocelluloses is without its own problems; it requires a microorganism that can secrete the enzyme - system in the right amounts for the complete hydrolysis and the creation of hydrolysis conditions to prevent end product inhibition of the enzyme - system either by cellobiose or glucose (Duff *et al.*, 1987 ; Holtzapple *et al.*, 1990).

The group of hydrolytic enzymes implicated in the bioconversion of celluloses are known as cellulases or cellulase-systems. The cellulase-system comprises three major highly specific enzymes namely; the endo-glucanases, the exo-glucanases and ß-glucosidases. These enzymes are non constitutive and are produced by many microorganisms such as bacteria, actinomycetes and fungi. The cellulase-systems of fungal origin are the most abundant and widely studied. Among the fungi, *Trichoderma*, a softwood rotting fungus, is the most potent cellulase-system producer.

The other problem, the fermentation of the sugars produced from the hydrolysis step, is plagued with problems ranging from the selection of yeast strains capable of fermenting mixtures of hexoses and pentoses (Grohmann, 1993), adapting yeast strains to fermentation conditions, and prevention of end-product inhibition by ethanol (Stokes, 1970).

Objectives of study

Several factors are implicated in the overall efficiency of ethanol production from lignocelluloses, namely, the source of cellulase and its quality characteristics, the cellulosic substrate and pretreatment method, the process for cellulase production and bioreactor design. Ethanol production through enzymatic hydrolysis of lignocelluloses is not presently economical since cellulases alone are estimated to be around 50-80% the cost of fuel ethanol production from lignocelluloses (Zacchi *et al.*,1988 ; Hendy *et al.*, 1984), thus it was decided that this work would concentrate on the optimization of the production of an effective cellulase-system, and its implications on hydrolysis of wheat straw and fermentation of its hydrolysates into ethanol. Thus, as shown in section 1-1, there were four main goals of this research.

The first goal was to develop an optimization model that describes optimum cellulase-system production with pretreated wheat straw in solid-state fermentation. Various pretreatments have been reported in the literature but it appears most have concentrated on the pretreatment of lignocelluloses for its hydrolysis. Even where the pretreatments have been used for cellulase-system production, different pretreatment levels have been reported for the same substrate mostly under liquid-state fermentation conditions and to a smaller extent on solid-state fermentation conditions. No model has been reported for the optimization of cellulase-system production with wheat straw pretreated with mild sodium hydroxide and steam explosion.

The second goal was to evaluate fermentation system designs for upgrading cellulase-system production under solid-state fermentation conditions. The larger question

answered here was how substrate concentration, and bioreactor design affect the growth characteristics of cellulase-system producers and their yields.

The third goal was to test two optimization models for optimum cellulose hydrolysis with both crude extracted and crude unextracted cellulase-systems from two mutants of *Trichoderma reesei*. Delignified wheat straw was chosen as the model cellulose substrate because it comes from the same substrate that was used for the cellulase-system production and also because the delignified wheat straw contained some cellulose and hemicelluloses. The experiments were, therefore, designed to obtain information on the hydrolytic potentials of different cellulase-systems in order to establish threshold values of substrate and cellulase-system loading that will maximize the formation of monomeric hexoses and pentoses. Since the ratio of the β-glucosidase activity to filter paper activity is important, experiments were also conducted to gain information about how these ratios affect the hydrolysis of delignified wheat straw. No such comparative study has been reported in the literature.

The fourth goal was to combine the cellulase-system production and hydrolysis models to describe separate hydrolysis and fermentation and simultaneous hydrolysis and fermentation systems. To this end, yeasts were selected that had the ability to ferment either glucose or xylose. Yield parameters such as efficiency of substrate conversion, ethanol production and productivity, and residual sugars were evaluated.

1.1 Objectives of study

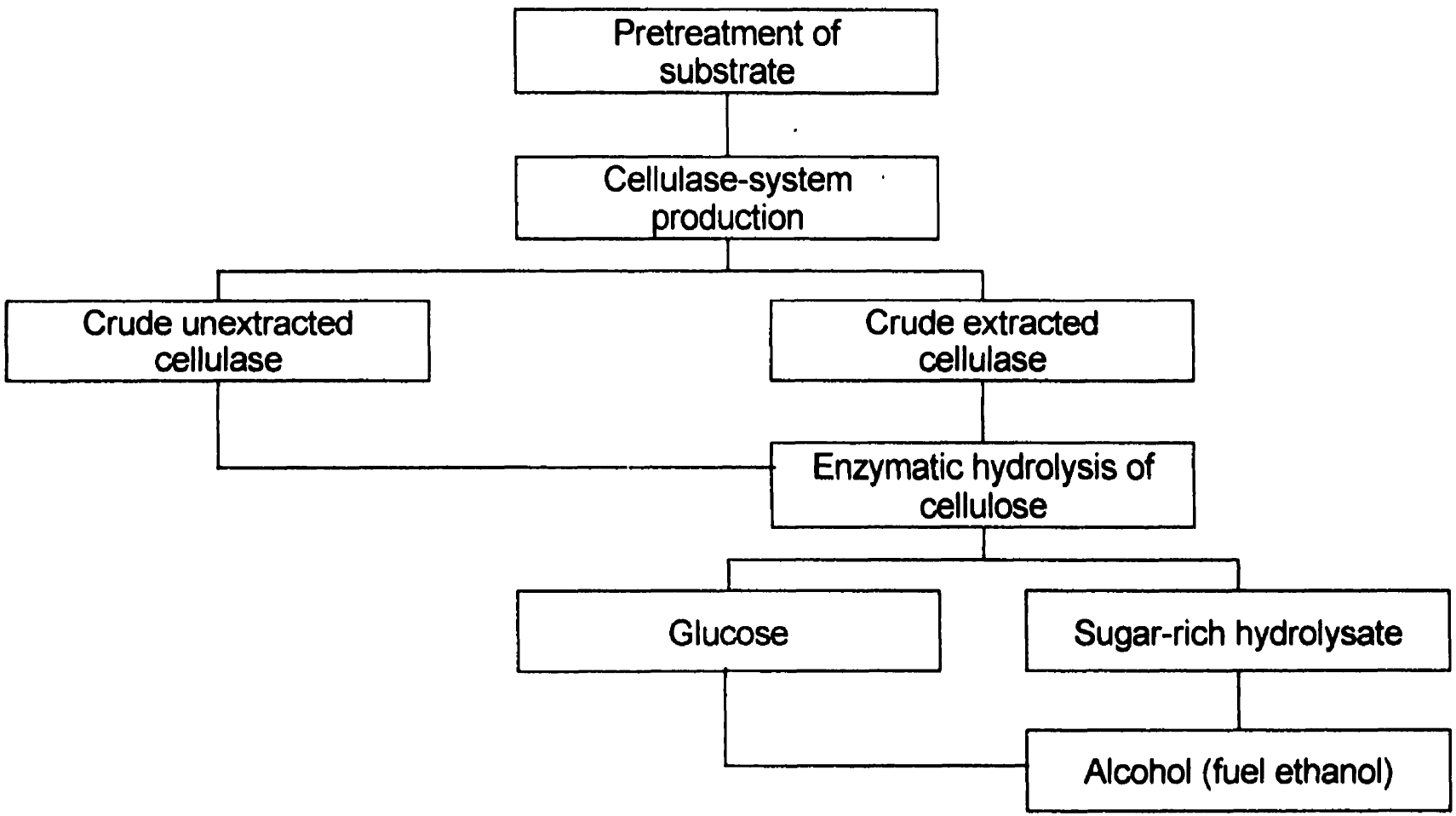
I. Develop models for optimum cellulase-system production with wheat straw vis à vis pretreatment type, fermentation system and cultural conditions.

II. Evaluate bioreactor design for cellulase-system production under SSF conditions.

III. Optimize cellulose hydrolysis with crude unextracted and crude extracted cellulase-systems of *T. reesei.*

IV. Evaluate ethanol production from cellulase-systems of *T. reesei* and their hydrolysates

Fig. 1-1 Biotechnological route for fuel ethanol production from lignocelluloses



Chapter 2

LITERATURE REVIEW

2.1 Background

Biotechnology has been defined as the use of whole cells or parts derived therefrom to catalyze the formation of useful products or to treat pollutants (McNeil and Harvey, 1995). Within the broad field of biotechnology, the filamentous fungi have long occupied an important position. This diverse and metabolically versatile group of eukaryotic microorganisms is characterized by the formation of vegetative branching filaments (hyphae), which together form a mycelium. Biotechnologically, the most important fungi are found within the Fungi Imperfecti, Ascomycetes, Basidiomycetes and some Phycomycetes. They are used in the service of humans to produce a wide range of valuable products, to improve feedstuffs, to carry out biotransformations, and to effect bioremediation. For instance, they are generally 50 -100 times more hydrolytic than the most active cellulolytic bacteria because they produce more cellulases (Saddler, 1986, Saddler *et al.*, 1984). *Trichoderma* belongs to the Fungi imperfecti (Deuteromycetes) and is able to grow on an inorganic medium supplemented with an organic carbon source to produce a cellulase-system capable of hydrolyzing cellulose into glucose.

The biosynthesis of cellulase-system is induced by cellulose and some other inducers and regulated via catabolic repression and their activity is influenced by end-product inhibition (Montenecourt and Eveleigh, 1977). The cellulase-system comprises the endo-

1,4-ß-glucanases, exo-1,4-ß-glucanases and ß-glucosidase and has direct applications in the food and pharmaceutical industries where it can be used for extraction processes, baking, malting, brewing, and grain alcohol production. The cellulase-system can, however, also be used for the enzymatic hydrolysis of pretreated lignocellulose into fermentable sugars (hexoses and pentoses) which can be subsequently fermented into ethyl alcohol. Ethyl alcohol produced from grains and sugar cane juice is already being marketed in many countries like Mexico, Canada, and USA as fuel ethanol in a 5-10% blend with gasoline, as an octane enhancer and fuel extender or used as a neat fuel in internal combustion engines (Emert, 1990 ; Wyman, 1994). In Brazil, it is used at 22-100% (Emert, 1990 ; Wyman, 1994).

The use of lignocellulosic substrates for fuel ethanol production could increase fuel flexibility, reduce the related strategic vulnerability of petroleum-based transportation fuel system, reduce the net accumulation of CO_2 in the atmosphere, and improve urban air quality. More still, lignocellulose has the eminent advantage, because of its low cost, of removing equivalent amounts of traditional sugars such as sugar cane, sugar beets and high-fructose corn syrups that are presently used for ethanol production, and making them more available for other uses. However, to compete with the price of petroleum, the cost of enzyme, the rate of hydrolysis, and product yield must be improved for enzymatic hydrolysis processes to be viable.

Cellulosic substrates have two potential advantages: less expensive equipment may be possible at the more mild reaction conditions, and higher recovery of fermentable sugars is possible since enzymes are highly specific.

2.2 Choice of Substrate for Cellulase-System Biosynthesis

There are two main characteristics to consider in choosing a substrate for cellulase-system biosynthesis. The first specific characteristic is that cellulase-system biosynthesis is not constitutive and therefore requires an inducer. The second characteristic is that cellulase-system biosynthesis suffers from end-product inhibition (Montenecourt and Eveleigh, 1977). To date, cellulose is the best known inducer. This makes lignocellulosic materials prime candidates for cellulase-system biosynthesis because of their universal abundance and high carbohydrate content (Table 2-1). Purified celluloses such as avicel and solka floc have drawbacks because they are quite expensive. Other substrates such as molasses cannot be used by *T. reesei* perhaps because they lack sufficient invertase activity. Glucose and fructose which are readily metabolized by fungi do not induce cellulase-system biosynthesis. Moreover, they are powerful repressors of cellulase-system biosynthesis through catabolite repression. Other substrates, like lactose, have been shown to be suitable substrate for cellulase-system biosynthesis (Allen and Moetensen, 1981). Lactose is an inducer of cellulase-system biosynthesis (Vandecasteele and Pourquie, 1984 ; Mandels *et al.*, 1962), is more slowly metabolized than glucose, and is less susceptible to catabolite repression. Lactose, however, also has severe drawbacks because it is not a readily available substrate, it has low solubility in water and since it is liquid, it has bulk handling problems.

2.3 Lignocellulosic Substrates

Lignocellulosic biomass can be obtained from many sources; traditional standing timber, underutilized forests, forest wastes, timber residues, agricultural residues and municipal wastes. In this group, standing timber is mainly used for lumber and pulp production and so it is the underutilized forests, forest wastes, agricultural residues, and municipal wastes that are largely underutilized and available as potential substrates for bioconversion into fermentable sugars for ethanol production.

Lignocellulosic materials are composed of carbohydrate polymers known as cellulose and hemicelluloses, plus lignin and small amounts of other extraneous materials and protein. Agricultural residues, municipal solid waste, underutilized standing forests and residues from logging operations, energy crops, such as short-rotation woody crops, and herbaceous crops, and waste streams from industrial operations are examples of this largely untapped source of renewable material.

It is estimated that about 50 billion tons of cellulose is produced annually world wide by photosynthesis (Dale, 1985) and at the same time, at least 12-20 billion tons of hemicelluloses are produced with an equal amount of lignin (Lieth, 1973)

2.3.1 Composition of Lignocelluloses

Cellulose is the most abundant naturally occurring organic compound in the plant world, and is defined in the 'wood' industry as a linear polymer of ß-1,4 D-glucose units which form long fibrils when held together by intramolecular hydrogen bonds (Darvill *et al.*,

1980). Attala (1983) describes the state of aggregation of cellulose as semi-crystalline solids and polymorphic crystalline solids. The ß-1,4 orientation of the glucosidic bonds results in the potential formation of six hydrogen bonds, four intramolecular and two intermolecular in the crystalline regions (Zhbankov, 1992). When all the six hydrogen bonds are formed, cellulose has a highly ordered, tightly packed structure which impedes acid or enzymatic hydrolysis. The different conformations of cellulose has implications in its enzymatic hydrolysis and may explain why organisms involved in cellulose hydrolysis have to synthesize and secrete multiple forms of the endo- and exo-cellulases for that purpose.

The controversial history of the term hemicelluloses has been described by Thompson (1983). Hemicelluloses are low molecular weight amorphous polysaccharides strategically located in the cell wall fiber. They are water-insoluble but are easily solubilized by alkali. Covalent bonds are thought to bind hemicelluloses chemically to lignin, and some chemical delignification treatments may even bind residual lignin to hemicellulose and cellulose by transglycosidation reactions. For convenience, hemicelluloses may be empirically classified as xylans, mannans, galactans, and galacturonans.

Lignin gives the plant cell wall rigidity and renders the lignocellulose complex relatively intractable. It is a polyphenol formed by enzyme initiated dehydrogenative polymerization of phenylpropanoid precursors (Sarkanen and Lugwig, 1971). Since the polymerization mechanism is enzyme initiated and free radical propagated, the resulting molecule is complex and seemingly random (Brown, 1964). The works of Morrison (1974) and Gaillard and Richards (1975) have shown alkali-labile bonding between lignin

and the structural polysaccharides and an ester linkage appears to be the most likely type of bond (Morrisson, 1973). Lignin in the cell wall, therefore, not only encrusts the cellulose microfibrils in a sheath-like manner, but is also bonded physically and chemically to the plant polysaccharide (Higuchi, 1971).

Table 2-1 shows the composition of selected lignocellulosic biomass.

Table 2-1: Composition of Some Lignocellulosic Biomass

Agro-residue	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Total weight accounted for (%)
Wheat straw	39	36	10	85
Rice straw	33	26	7	66
Barley straw	44	27	7	78
Guar straw	28	23	16	67
Maize straw	32	28	13	73
Cane bagasse	33	22	14	69
Oat straw	41	16	11	68
Hardwood	45.8	30.7	20.3	96.8
Softwood	43.8	24.5	29.5	97.8

Adapted form: Staniforth, 1979; Parisi, 1989.

Table 2-1 shows significant amounts of cellulose and hemicelluloses that are the sources for the production of cellulase-systems and their hydrolysis into fermentable sugars. The cellulose, hemicelluloses and lignin do not account completely for the composition of the lignocellulosic residues since extractives, such as, waxes, starches, oils, gums and cytoplasmic constituents account for part of the difference. Non-extractives such as silica, oxalates, alkali, and non-cellular substances account for an additional 10% of the material weight of straws (Ladisch and Svarczkopf, 1991).

Fig. 2-1 shows the structure of the cellulose component of lignocelluloses while Fig. 2-2 (a and b) show how the three main lignocellulose components are structurally linked in plant materials.

The cohesion of the plant cell wall is primarily due to the presence of its principal components: a crystalline polymer, cellulose, and a three-dimensional macromolecule and lignin. These components are embedded in a matrix of pectic and hemicellulolytic polysaccharides of various nature. It is generally accepted that the relations that exist between these different polymers are established through linkages of different chemical nature. For instance, blocks of lignin are associated through hemicellulose chains. The hemicellulose, another major component of lignocellulosic material, consists largely of 4-O-methylglucuronoxylan that also includes the β-1,4-linked polymer of D-xylose.

2.4 Substrate Pretreatments

The polysaccharides (cellulose and hemicelluloses) are held together by intermolecular hydrogen bonds while covalent bonds bind hemicellulose chemically to lignin (Fig. 2-2)

Pretreatment of lignocelluloses is aimed at modifying the structural characteristics of the lignocellulosic matrix and disruption of the cellulose-hemicellulose-lignin seal for subsequent chemical or enzymatic hydrolysis and/or for fermentation for the production of cellulase-systems. This is necessary because crystalline cellulose and encrustation of cellulose fibrils with lignin make the lignocellulose recalcitrant. Once lignin is depolymerized, solubilized or removed, cellulose and hemicelluloses can either be easily hydrolyzed enzymatically into fermentable sugars for the production of ethanol or used as fermentation substrate for the production of cellulase-systems.

Pretreatment methods cited in the literature are more often used for rendering substrate susceptible to hydrolysis by cellulase enzymes. Substrate pretreatment is, however, also a requisite for cellulase enzyme production.

Several different pretreatment methods have been used to enhance the rate of hydrolysis of lignocelluloses. Such methods include, physical (Chang *et al.*, 1981, Gharpuray *et al.*, 1983), chemical (Rolz *et al.*, 1987) and biological (Hatakka, 1983). Some of the physical or mechanical methods include: ball milling (Millet *et al.*, 1979), attrition milling (Ryu and Lee, 1983), steam explosion (Brownell and Saddler, 1987), and irradiation (Han and Ciegler, 1982). Chemical pretreatments such as batch acid prehydrolysis (Wilke *et al.*, 1981), caustic treatment (Novald *et al.*, 1977), and solvent delignification (Avgerionos *et al.*, 1981) have been used to improve subsequent hydrolysis.

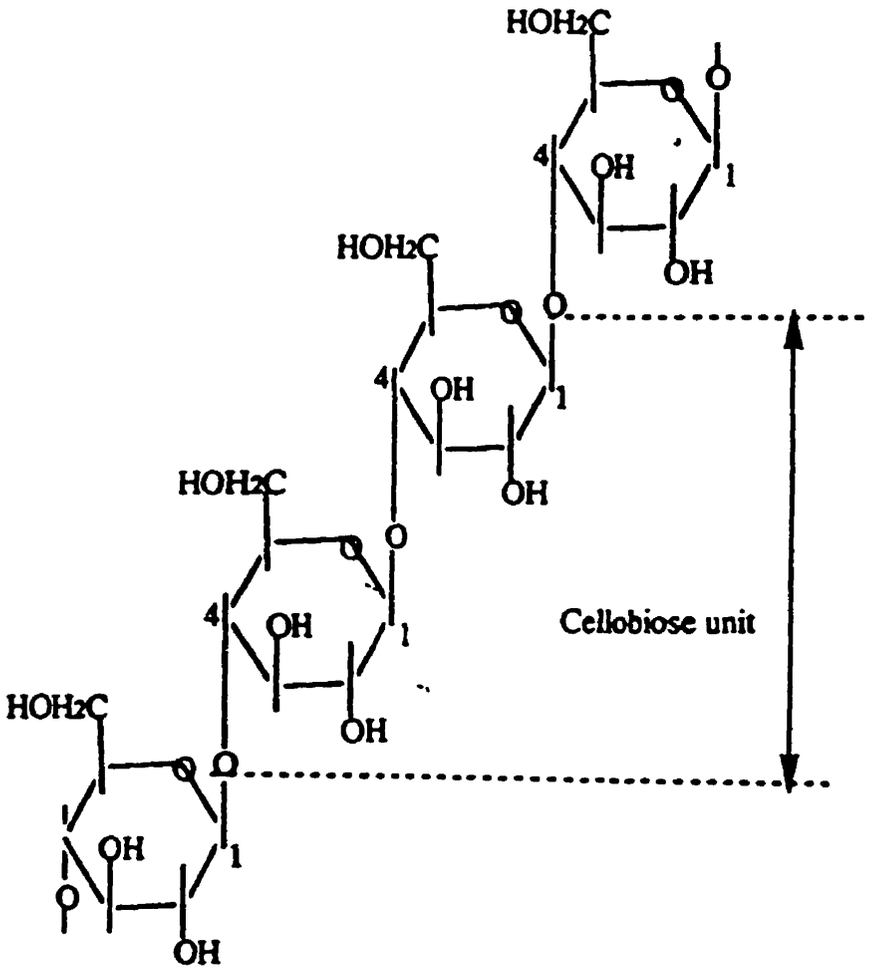
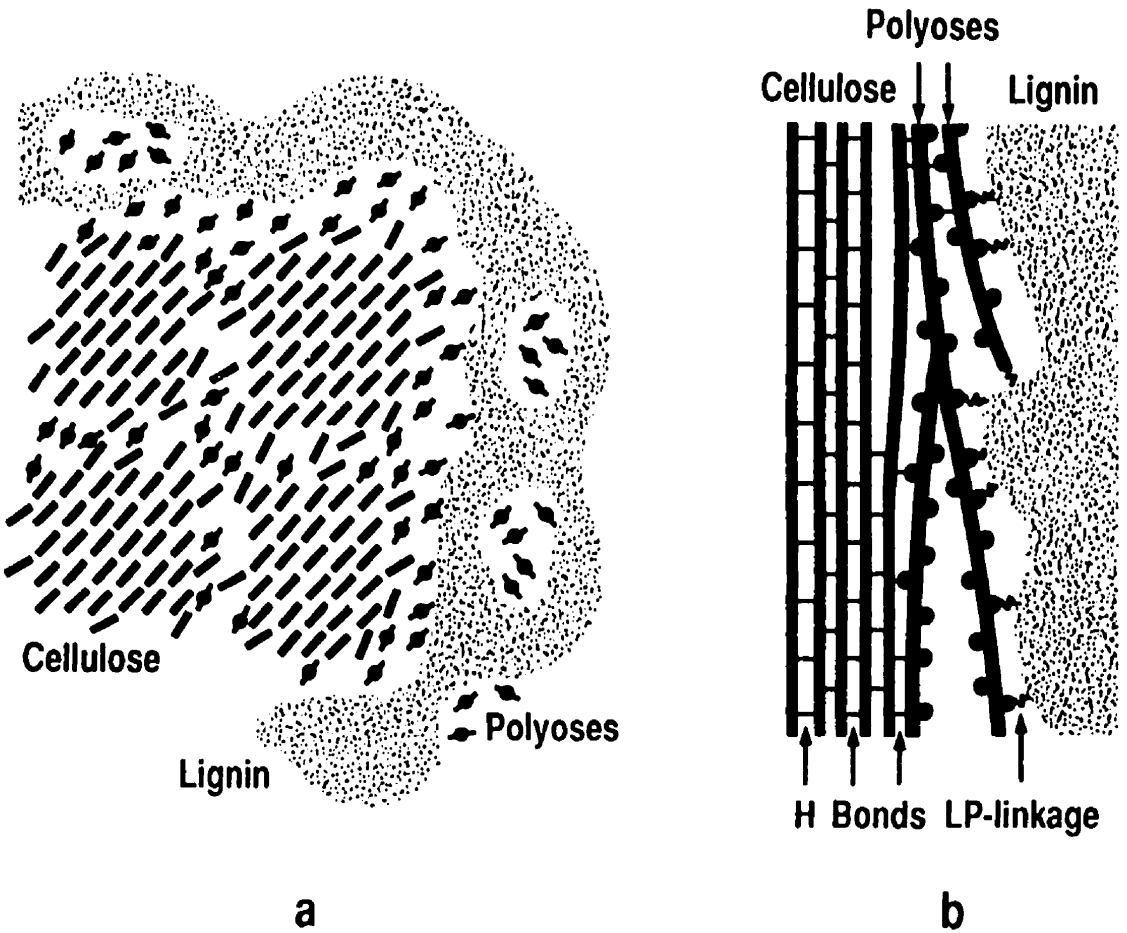


Fig. 2-1 Sketch of cellulose chain made up from glucose molecules linked through C-1 and C-4 positions. Subunits of two glucose molecules are considered as cellobiose units. (Reproduced from Adam (1983) I.A.E.A. Panel Proceedings Series).

Fig. 2-2 Structural organization of the three main components of plant materials (Fengel and Wegener, 1983)



2.4.1 Milling

Milling lignocellulosic material is a popular method for increasing cellulose digestibility (Tassinari *et al.*, 1982). The shearing and compulsive forces generated by the mill for a period of time on the material brings about a reduction in crystallinity, increase in the fraction of the material that is water soluble and decrease in particle size (Lyons and Kelsall, 1991). According to Mandels *et al.*, (1974), mechanical milling is effective in disrupting crystallinity of cellulose. It also increases the susceptibility of both amorphous and crystalline portions of cellulose. They found that the enzyme digestibility of the crystalline portion was enhanced to a greater extent by milling than that of the amorphous portion. This led them to propose that the overall increase in the digestibility is apparently as a result of decreased particle size and increased available surface area rather than as a result of reduced crystallinity. The other advantage of milling is that it is relatively substrate insensitive but the major drawback is that it requires high energy inputs and yet at the end of milling, lignin still remains a substantial barrier.

2.4.2 Alkali Pretreatment

Dilute alkali increases the fiber saturation point and the swelling capacity of lignocelluloses. The increase in swelling capacity results from the saponification of esters of 4-O-methylglucuronic acid attached to xylan chains. In the native state, the esters act as cross links, limiting the swelling capacity or dispersion of polymer segments in water. Such treatments increase the accessibility of plant polysaccharides to cellulolytic

microorganisms (Tarkow and Feist, 1969). Both alkali-labile and alkali resistant lignin-carbohydrate bonds exist and their ratio in a particular plant material governs the decision to use alkali as a pretreatment to increase its digestibility. The alkali-labile bonds include the hemicellulose-phenolic acid and acetyl constituents of cell walls (Hartley, 1981) both of which affect holocellulose hydrolysis (Jung and Fahey, 1983).

Alkali concentration of 0.1 g/g substrate has been reported by Choudhurry *et al.*, (1984) and Gray *et al.*, (1978) to be optimum for significant increases in the rate and extent of hydrolysis of lignocelluloses. When corn husk were treated between 25°C and 85°C with 1.25 M NaOH, 60.6% of the lignin and 71.6% of the hemicelluloses were solubilized. The extent of enzymatic conversion of cellulose to glucose has been reported to vary with the NaOH concentration used to pretreat the substrate (Koullas *et al.*, 1992). Conversion was under 30% for 2-4% NaOH, whereas 8-10% NaOH resulted in conversions between 80% and 90%.

The pretreatment of mixed hardwood (90% birch and 10% maple) using 2.5% (w/v) hydrogen peroxide at 25°C and pH 11.5, for a residence time of 19 h resulted in 68% glucose yield. The alkaline peroxide showed a moderate removal of lignin and hemicelluloses, a large increase in the swelling and pore volume of cellulose, and a decrease in the cellulose crystallinity index. Alkali pretreatment in combination with irradiation has also been explored by Awafo *et al.*, (1995) to study its effect on the structural modification of corn stover for mycelial biomass protein production. The lignin and hemicelluloses not removed by any pretreatment remains largely associated with the cellulose and may hinder the enzymatic hydrolysis of the latter (Thompson *et al.*, 1991)

2.4.3 Hydrothermal Pretreatments

Lignocelluloses can be pretreated hydrothermally by steam alone, acid steam pretreatment or alkali steam pretreatment.

2.4.3.1 Steam Treatment With or Without Addition of Acid/Alkali

Steam explosion is a thermomechanical process in which the lignocellulose can be separated into three fractions by rapidly heating lignocellulosic material in a sealed vessel and then explosively releasing the fiber-steam mixture through a nozzle. The steam explosion parameters that have generally been employed for the pretreatment of lignocelluloses, namely, aspen, eucalyptus, spruce, pine wood chips, and wheat straw had steam in the range of 250-650 psig at temperatures in the range of 200°C to 240°C for 30 s to 20 min (Ramos *et al.*, 1992 ; Schell *et al.*, 1991; Brownell and Saddler, 1987; Beltrame *et al.*,1992). Steam treatment under high pressure (autohydrolysis) makes the lignocelluloses easily accessible to hydrolytic enzymes (Noble, 1980)

Steam treatment was originally developed in 1925 and has been extensively used in the manufacture of hardboard by the Masonite process employing wood. In the late 70s, Iotech Corp. Ltd. and Stake Technology Ltd. in Canada started using this process for the production of feed for ruminants.

Studies of steam pretreatment have shown that the lignocellulosic matrix of wood is modified drastically and the remaining solids are more susceptible to enzymatic hydrolysis. The structural changes and the chemical reactions taking place seem to be a

function of temperature and time of pretreatment. The hemicelluloses are hydrolyzed to soluble sugars by organic acids, mainly acetic acid derived from acetylated polysaccharides present in wood (Casabieci *et al.*, 1969). Under more drastic conditions, however, secondary reactions occur which result in the formation of furfural, hydroxymethyl furfural and their precursors by dehydration of pentoses and hexoses. Campbell *et al.*, (1973) reported that phenolic-like compounds increased from 0.43 to 4.5% in steam-pretreated bagasse at 500 psig for 45 min. The residence time at higher temperatures is kept low to minimize reactions which produce inhibitory byproducts (Neese *et al.*, 1977). Beltrame *et al.*, (1992) reported that longer time and higher temperature pretreatments of wheat straw resulted in lower overall recovery of dry matter ranging from 46-90 % and indicated that significant autohydrolysis and degradation of sugars can occur during pretreatment.

According to MacDonald and Mathews (1979), steaming biomass leads to increased enzymatic digestibility and the increased pore size, autohydrolysis and wetting allow the enzymes to penetrate further and increase bioconversion yields substantially over the non-pretreated material.

Steam explosion has also been used to pretreat wheat straw, bagasse, and eucalyptus wood chips to obtain maximum glucose yields of 81, 78, and 75%, respectively (Puri and Mamers, 1983). Mes-Hartree *et al.*, (1983) also used steam explosion to pretreat barley straw, wheat straw, corn stover, corn stalks, and alfafa stalks to amend them for enzymatic hydrolysis. Moniruzzaman (1996) obtained glucose yields of 76% with steam exploded rice straw and a saccharification efficiency of 92% with the unfractionated steam exploded rice straw.

Ramos *et al.*, (1992) and Schell *et al.*, (1991) have reported that steam pretreatment of lignocelluloses is enhanced when sulfur dioxide (SO_2) is added to wood chips. The SO_2 diffuses into the wood as sulfurous acid (HSO_3) which then turns into sulfuric acid (H_2SO_4) during the steaming process.

2.4.4. Biological Delignification of Lignocelluloses

Lignin-utilizing organisms or their extracted cellulase-systems have been used for the biological pretreatment of lignocellulosic materials. Detroy *et al.*, (1980) used *Pleurotus ostreatus* to partially delignify wheat straw and to increase enzymatic saccharification after 50 days of fermentation. The lignolytic white-rot fungus *Phanaerochaete chrysosporium* has been used to pretreat wood chips to make them more amenable to pulping and saccharification (Eriksson *et al.*, 1980).

Several authors have also used hemicellulases (Ghose and Bisaria, 1979; Schwald *et al*, 1984), and pectinases (Beldman *et al*, 1984; Coughlan *et al*, 1985) in conjunction with cellulases to increase the extent of conversion of lignocellulosic materials.

Several reviews (Millet *et al.*, 1975, Chang *et al.*, 1981; Fan *et al.*, 1981) have covered methods for increasing the availability of cellulose and most have categorized them into physical, chemical, biological, and combinations of these methods (Table 2-2).

Table 2-2: Major Methods for Pretreating Lignocellulosic Materials to Enhance Enzymatic Hydrolysis

Physical	Physico-chemical	Chemical	Biological
Irradiation	Steaming	Oxidation (H_2O_2)	White rot fungi
Pyrolysis	Autohydrolysis	Gases (SO_2, NO_2)	Enzymes
Ball milling	Ammonia explosion	Cellulose solvents	
Hammer milling	Wet oxidation	Ammonia	
Extrusion		Acid/Alkali	
Wetting		Organic solvents	

Source: Goldberg (1985)

2.5 Effectiveness of Pretreatments.

The effectiveness of various pretreatments has been attributed to lignin removal, reduction in crystallinity (Sattler *et al.*, 1989; Vallander and Eriksson, 1990), increase in total surface area, and increase in the pore volume and corresponding surface area available to the enzyme (Grethlein, 1985 ; Thompson and Chen, 1992). The contribution of some of these factors on cellulose hydrolysis is, however, controversial in some pretreatments. Grethlein and Converse (1991) have reported that lignin may not have to be removed in order to attain high rates of cellulose conversion by acid and steam pretreatment. However, in the hydrolysis of lignocelluloses, the lignin content of the substrate will likely affect the recovery of cellulases during hydrolysis. It has been shown that cellulases adsorb to both isolated lignin (Chernaglazov *et al.*, 1988), and the lignaceous residues remaining after complete hydrolysis of the cellulose component (Girard and Converse, 1993 ; Ooshima *et al.*, 1990).

The crystallinity index as measured by X-ray diffraction has also been reported to be of no major importance in steam and acid pretreatments (Ryu *et al.*, 1982; Betran and Dale, 1985). Saddler *et al.*, (1982), Dekker and Wallis (1983) also reported that the crystallinity index remained the same or increased slightly with steam and acid. Recent work by Pulls and Wood (1991) has also shown that cellulose crystallinity is relatively invariant over extended hydrolysis time.

2.6 The Microorganism

Trichoderma species are soil fungi that are placed in a super-kingdom based in part on their absorptive metabolism (Whittaker and Margulis, 1978). They occur ubiquitously

and excrete extracellular enzymes like cellulases. Fig.2-3. shows the genealogy of *Trichoderma* species used for the production of cellulase-systems in this work. *Trichoderma* was first described by Persoon in 1794, but the classic cellulase producing *Trichoderma* strain from the U.S Army Natick laboratories was described by Simmons (1977) and named it as *T. reesei.*

Even though the best mutant strains can now secrete up to 40 g L^{-1} extracellular protein, most of which is cellulase, the proportions of the individual cellulase components and their specific activities have remained practically similar to those of the initial parent strain (Allen and Roche, 1989).

2.7 The Lignocellulosic Enzyme-System

Pretreated lignocellulosic material requires two enzyme-systems for its hydrolysis; the cellulase-system and hemicellulases. Hemicellulases, xylanases being dominant, hydrolyze hemicelluloses into its monomers like xylose, mannose, arabinose and galactose (Rapp and Wagner, 1986; Wong *et al.*, 1986). The cellulase-system comprises of a complex hydrolytic mixture of enzyme proteins that act synergistically with different specificities to hydrolyze glycosidic bonds in crystalline cellulose to glucose. The cellulase-system is termed 'complete' when the ratio of ß-glucosidase activity (ßGA) to that of the total activity, expressed as filter paper activity (FPA), is close to one. This relationship is important in alleviating cellulose hydrolysis of feedback inhibition of cellobiose.

The cellulase-system of *Trichoderma* is the best characterized and consensus of the cellulase components of *Trichoderma reesei* based on biochemical and genetic criteria include:

i. Endo-cellulase (1,4-ß-D-glucan-4-glucanohydrolase, also known as endoglucanase, EG). It has the numerical designation as E.C. 3.2.1.4. It hydrolyzes amorphous or soluble substituted derivatives of cellulose in a random manner.

ii. Exo-cellulase (1,4-ß-D-glucan cellobiohydrolase, also known as cellobiohydrolase, CBH or exoglucanase, E.C. 3.2.1.91). They release cellobiose and some glucose units from the non reducing ends of the cellulose chains.

Synergy between these enzymes has resulted in significant increase in the rate of hydrolysis of crystalline cellulose upon addition of trace amounts of endoglucanase to a mixture of exoglucanases (Fägerstam and Pettersson, 1980 ; Wood *et al.*, 1989), and

iii. ß-glucosidase (ß-D-glucoside-glucohydrolase also known as cellobiase or ß-G) has the designation, EC. 3.2.1.21. It releases glucose from oligomers (cellobiose to cello-octaose) (Schmid and Wandrey, 1987). Another function of ß-glucosidase may be to use glucose (via a transglycosylation reaction) to produce oligosaccharides that have been shown to act as potent inducers of the cellulase-system (Vaheri *et al.*, 1978; Kubicek, 1987).

Sequence comparisons of cloned cellulolytic enzymes (Saloheimo *et al.*, 1988), and biochemical data (Van Tilbeurgh *et al.* 1986 ; Tomme *et al.*,1988) show that *T. reesei* has two distinct cellobiohydrolases, CBH I and CBH II. CBH I is the dominant enzyme forming about 60% of the total secreted proteins. It has a molecular weight ranging from 42-72 kDa and a carbohydrate content of 1.4 - 10.4%. It also has 497 amino acid residues (52214 Da), 12 disulphide bridges and no free cysteine residues. CBH II forms about 20% of the secreted proteins with molecular weight ranging from 50-58 kDa and shows about 18% glycosylation. Three endoglucanases have been recognized, EG I, EG II, and EG III. EG I is dominant forming 6-10% of the secreted protein with molecular weight of 54 kDa and has about 4% carbohydrate content.

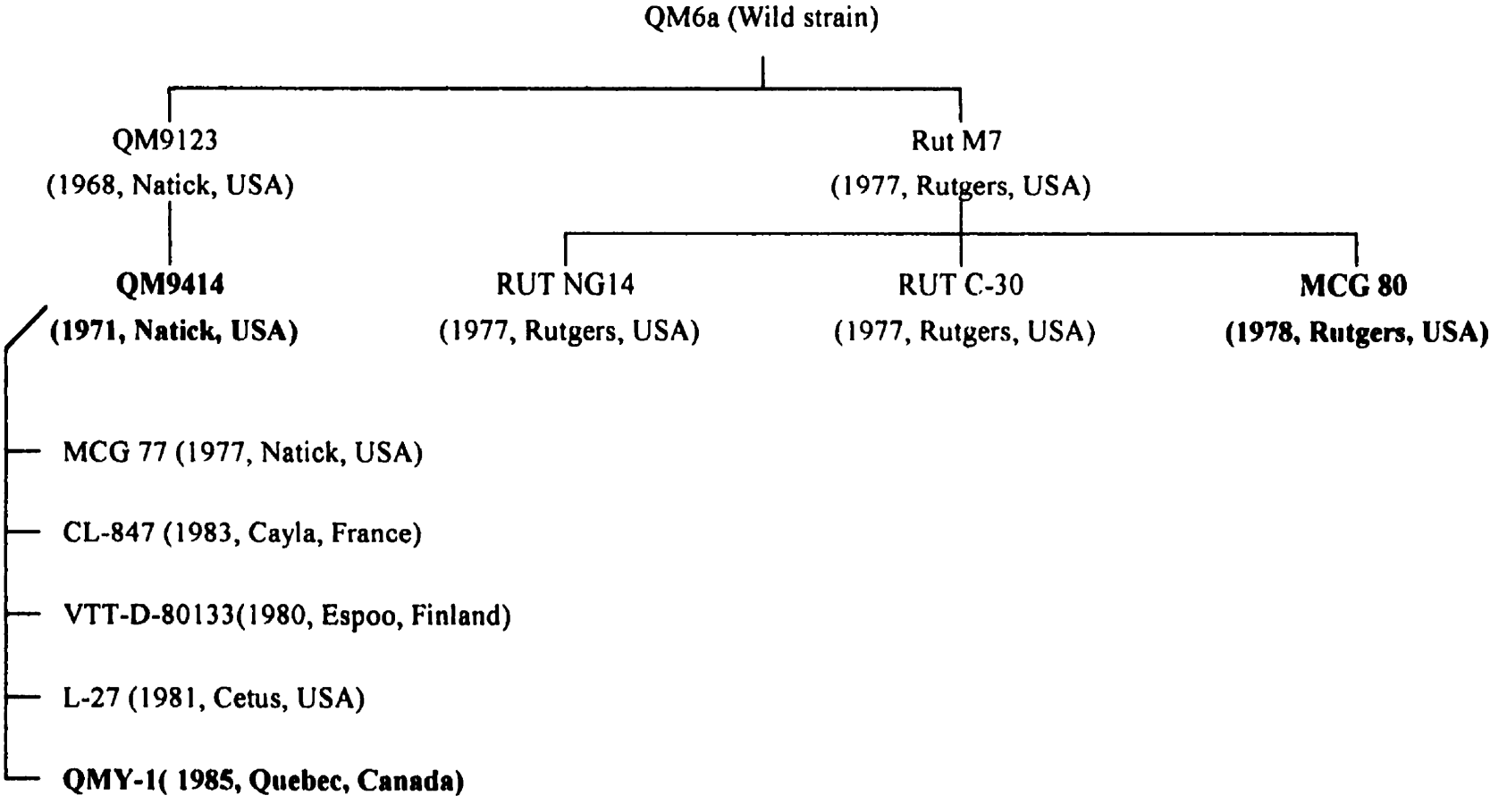
Cell fractionation, immunoflourescence and electron microscopic localization indicate that the majority of ß-glucosidase is associated with the outer interguments of *T. reesei* (Usami *et al.*, 1990), and comprises about 1% of the secreted protein. The association of ß-glucosidase with the cell wall may influence the ability of commercial preparations of cellulase to produce glucose.

Fig. 2-4 shows a schematic representation of the structure of *Trichoderma reesei* cellobiohydolase I based on small angle X-ray scattering (Schmuck *et al.*, 1986, Teeri *et al.*, 1992). The X-ray scattering data of *T. reesei* cellobiohydrolase I (Schmuck *et al.*, 1986; Abuja *et al.*, 1988), indicate that CBH I is tadpole shaped and 18 nm long.

The structural organization of one group of cellulases from both fungal and bacterial origin, revealed by limited proteolysis with papain, shows three distinct regions; the active site, the binding site, and hinge region (Van Tilbeurgh *et al.*, 1986; Tomme *et al.*, 1988). The active site, also known as the core or catalytic region, is located in the main

part of the enzyme and has a short extra binding domain connected to the core region via a flexible arm or hinge region. This organization improves binding to the substrate and, therefore, hydrolysis of crystalline cellulose.

Fig 2-3 Genealogy of *Trichoderma reesei* mutants used in study.



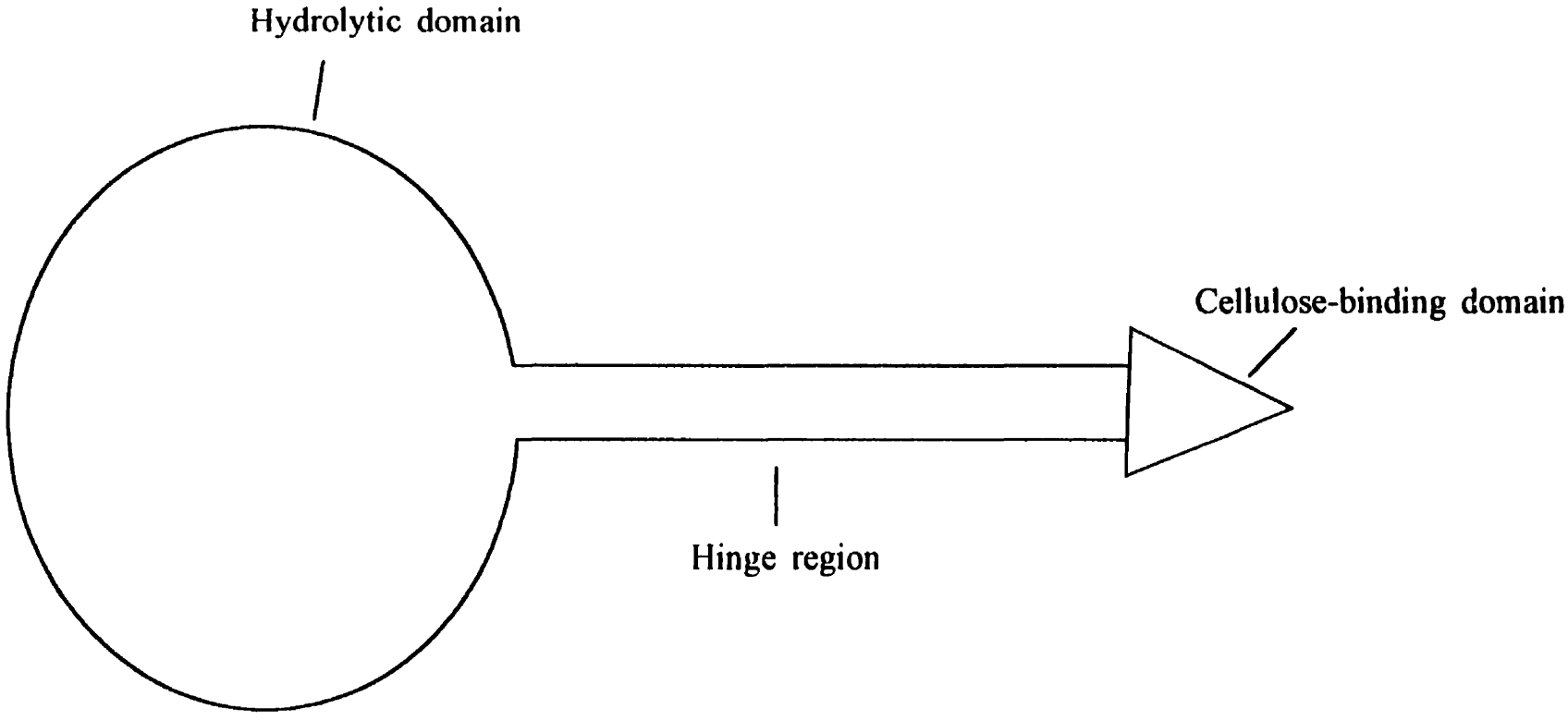


Fig.2-4: Schematic representation of the domain structure of *T. reesei* cellulases (Teeri *et al.*, 1992)
(Not drawn to scale)

2.8 Production and Applications of Cellulase-Systems- the incomplete story

Cellulase-systems are produced under liquid (submerged) state fermentation (LSF) or solid-state fermentation systems (SSF) with insoluble substrates such as pure cellulose or pretreated lignocelluloses. In SSF, the insoluble substrate is fermented with sufficient moisture, but without free water while in the LSF, the insoluble substrate is fermented in a slurry of 1-5% suspension (Chahal, 1985).

It is difficult to compare cellulase production values in the literature even for the same substrate and micro-organism because of variations in fermentation conditions especially as they relate to methodologies and reference samples. However, Tables 2-3 and 2-4 show examples of the cellulase activities obtained under the experimental conditions employed by the various researchers.

Table 2-3: Cellulase-System Production by Fermentation of Pure Celluloses with Some Mutants of *T. reesei*

Strain	Substrate	Fermentation type	FPA (IU/mL)	ßGA (IU/mL)	Ratio of ßGA: FPA	Yield (FPU/g)	Reference
QM6a	6% roll-milled cotton	batch (LSF)	0.7	0.04	0.06	83	Ryu and Mandels, 1980
QM9414	6% roll-milled cotton	batch (LSF)	0.7	0.04	0.06	167	Ryu and Mandels, 1980
QM9414	3% Cellulose	batch (LSF)	7.2	-	-	178	Mukhopadhay and Malik, 1980
Rut NG14	6% roll-milled cotton	batch (LSF)	0.7	0.03	0.04	250	Ryu and Mandels, 1980
Rut C-30	5% Solka Floc	batch (LSF)	0.7	0.35	0.5	-	Pourquie *et al.*, 1983
MCG 77	6% roll-milled cotton	batch (LSF)	0.8	0.06	0.075	-	Pourquie *et al.*, 1983
RL-P37	5% Solka Floc	batch (LSF)	1.3	0.25	0.19	-	Sheir-Neiss and Montenecourt, 1984

Table 2-4: Cellulase-System Production by Fermentation of Lignocelluloses and Other Waste Materials with Some Mutants of *T. reesei*

Strain	Substrate	Fermentation type	FPA (IU/mL)	ßGA (IU/mL)	Yield (FPU/g)	Reference
SVG-17	5% wheat straw	Fed batch (LSF)	6.5	-	-	Shoemaker *et al.*, 1981
Cl-847	6% lactose + 0.5% pulp	batch (LSF)	8.6	6	143	Pourquie and Desmarquest, 1989
Rut C-30	9% steam exploded aspen wood	batch (LSF)	7	-	78	San Martin *et al.*, 1986
MCG 80	5% lactose	Continuous	6.0	-	120	Allen and Andreotti, 1982
MCG 77	3% steam exploded wheat straw	batch (LSF)	2.4	-	147	Doppelbauer *et al.*, 1987
MCG 77	2% sulfite pulp	batch (LSF)	3.7	-	185	Doppelbauer *et al.*, 1987

LSF = Liquid state fermentation.

2.8.1 Solid-State Fermentation

The relationship between microorganisms and their animal and plant hosts is lost in antiquity and so does the origins of solid-state fermentation. This method has been used for the production of aflatoxins (Hesseltine, 1965; Shotwell *et al.*,1966) and for upgrading the nutritional value of foods especially oriental foods (Hesseltine, 1965). Solid- state has also been used for the bioconversion of lignocelluloses into protein-rich animal feed. It has also been used for the production of amylases and cellulases (Toyama, 1976). SSF has of late been of interest in cellulase-system production because of the high cellulase yields obtained by various researchers (Chahal, 1985 ; Chahal *et al.*, 1996).

SSF has some distinct advantages over LSF in the production of cellulase-systems.

i. The cellulase-system produced by LSF is normally deficient in the ß-glucosidase and is therefore not termed as a complete cellulase-system. Fermentation systems employing this technology always require an external sources of ß-glucosidase (Stockton *et al.*, 1991; Wood, 1977)

ii. Higher cellulase activity can be obtained with SSF at a far less cost of filtration than with LSF

iii. Solid unextracted cellulase-systems can improve cellulose hydrolysis because of increased availability of ß-glucosidase associated to cells. Unextracted cellulase-systems from SSF also have the tendency not to dilute the hydrolysis or fermentation medium compared to unextracted cellulase-systems from LSF

However, LSF has the distinct advantage of effective process controls and less chances of contamination because of the short duration of fermentation times.

2.8.2 Improvements in the Activity of Cellulase-Systems- An Update

Several methods have been employed to improve the activity of cellulase-systems especially as they relate to *T. reesei*. Most of the improvements so far have been based on either increasing the total cellulase activity or the ß-glucosidase component of the cellulase-system. To increase the total cellulase-system activity and specifically the ß-glucosidase activity, the following approaches have been employed and are still being improved upon;

i. Optimization of media and fermentation conditions under mostly liquid fermentation systems. A few reports have, however, paid some attention to improving solid-state fermentation systems for cellulase-system production (Chahal,1991, Toyama, 1976).

ii. Screening of cellulase producing mutants: This technique has resulted in many hyperproducing mutants in which enzyme synthesis is less subject to catabolite repression and/or in which some of the cellulase-system components are less inhibited by the products of cellulose hydrolysis (Waldron *et al.*, 1986; Durand *et al.*, 1988).

iii. Recombinant DNA methodology has allowed the cloning and expression of cellulolytic genes in non-cellulolytic hosts. Cloning and sequencing defines the amino acid sequence of the cellulase-system and allows its over production in the case of total cellulase activity or for mutagenesis to determine the role of specific amino acids

residues in the function of the enzyme i.e. ß-glucosidase. Cellulases have been cloned from *Trichoderma* such as *Trichoderma fusca* (Lao *et al.*, 1991 ; Hu and Wilson, 1988). The role of ß-glucosidase genes from *T. reesei* are also investigated at the molecular level (Barnett *et al.*, 1991).

iv. Mixed culture cultivation. This technique involves the cultivation of *T. reesei* with an effective ß-glucosidase producer (Duff *et al.*, 1987 ; Ghose *et al.*, 1985)

2.8.3 Potential Applications of Cellulase-systems

Cellulases produced by *T. reesei* are presently used in starch processing, animal feed applications, grain alcohol fermentation, malting and brewing and extraction of fruit vegetable juices (Mandels, 1985). Other applications include their use in different processes of the pulp and paper industry such as in the improvement of the quality of the mechanical mass, pulp milling, and paper mill wastewater treatment (Linko *et al.*, 1989). Cellulases are also used for upgrading garment washing techniques by using them to replace or reduce the amount of stones used in jeans manufacturing (Tyndall, 1990).

Cellobiohydrolases are quite adequate for direct application in yeast for the preparation of beverages (glucan haze removal) and the preparation of food products with modified texture (Goyal *et al.*, 1991).

The glucose obtained from the enzymatic hydrolysis of cellulose can serve as raw material for fermentation products such as microbial biomass protein and antibiotics (Linko, 1977, Mandels, 1985).

2.8.4 Constraints in the Production of Cellulase-systems

The major constraints in cellulase-system production with lignocellulosic material are substrate concentration and environmental controls. Whereas SSF can accommodate high substrate concentrations, LSF is limited to substrate concentrations up 5% because of agitation, aeration and mass flow problems. SSF on the other hand suffers from environmental controls of pH because of sustained long periods of incubation with no chance of effective pH controls.

2.8.5 Measurement of the Activity of Cellulase-Systems - the Reference Substrate

Classical substrates used in the measurement of cellulase-system activities include filter paper, Avicel, carboxymethyl cellulose (CMC), and α-cellulose (Solka floc). Filter paper is used to assay for the 'total cellulase-system', i.e., exo-glucanase, endo-glucanase, and ß-glucosidase. Avicel and CMC, on the other hand, are used to measure the exo-glucanase and endo-glucanase components of the cellulase-system. Endoglucanase can also be assayed by using soluble substituted cellulose such as non-ionic hydroxyethyl cellulose (Child *et al.*, 1973).

Problems with some of these substrates include the non-linearity of the 'dilution curve', i.e., linear activity (absorbancy) versus time curves (initial rates) for CMC (Lindner *et al.*,

1983). Some authors have used chromogenic (fluorogenic) glycosides as alternative substrates for cellulases (Van Tilbeurgh *et al.*, 1982 ; Claeyssens and Aerts, 1992). However, the International Union of Pure and Applied Chemistry (IUPAC) has recommended the use of filter paper as substrate for the determination of total cellulase activity based on 4% conversion under specific digestion conditions (Ghose, 1987). In the determination of ß-glucosidase activity, cellobiose, salicin or chromogenic ß-glucoside are all recommended substrates (Mandels *et al.*, 1976 ; Ghose, 1987 ; Goyal *et al.*, 1991 ; Breuil *et al.*, 1992).

2.9 Cellulose Hydrolysis - the Models and their Merits

Structural cellulose is a crystalline polymer associated in a matrix with lignin and hemicellulose and as such is highly resistant to enzymatic attack. Cellulose is a heterogeneous porous substrate and its rate of hydrolysis is governed by the number of glucose residues that are accessible to the rather large cellulase enzymes. In hydrolysis, effective pretreatment should increase the number of available sites for cellulase action. Cellulose hydrolysis involves basically two steps; adsorption of cellulase onto the surface of cellulose and breakdown of the cellulose into fermentable sugars (Ryu *et al.*, 1984 ; Lee and Fan, 1982). The detailed steps leading to cellulose hydrolysis are as follows:

i. Diffusion of the cellulase-system from the bulk of fluid to the film layer immediately adjacent to the solid cellulose particle
ii. Adsorption of the cellulase-system onto available sites on the cellulose particle
iii. Formation of cellulase-substrate reactive complex

iv. Hydrolysis of the cellulose polymer's glycosidic bond

v. Diffusion of the hydrolysis product away from the cellulase-substrate active site and into the bulk fluid

vi. De-adsorption of the cellulase-system from the now reacted site.

The adsorption of cellulase is a central feature of several models pertaining to the enzymatic hydrolysis of cellulose. Enzyme adsorption is strongly influenced by the physico-chemical properties of the substrate (Ooshima *et al.*, 1983; Lee and Fan, 1982), the nature of the cellulase-system (Klysov *et al.*, 1986), and physical reaction parameters such as temperature (Ooshima *et al.*, 1983) and mass transfer (Sakata *et al.*, 1985). According to Cowling (1975), the most important feature that influences the enzymatic hydrolysis is the accessibility of cellulose surface to the cellulase-system and direct physical contact between the enzyme molecules and the substrate cellulose is a pre-requisite to hydrolysis. In cellulosic hydrolysis, the cellulase must be able to contact the glucosidic bonds (Grethlein and Converse, 1991)

The elucidation of the mechanism of action of the cellulase-system in the hydrolysis of cellulose requires the understanding of three different phenomena (Woodward, 1991).

i. The nature of the amino acids in the catalytic site of the individual cellulase-system components

ii. The mechanism of the binding of the cellobiohydrolase (CBH) and endoglucanase (EG) components to the surface of the insoluble cellulose

iii. The cooperative behavior or synergism that occurs between the CBH and EG to bring about the complete hydrolysis of cellulose to glucose.

Although the nature of the amino acids involved in the catalytic activity of cellulase components are not known, it is likely that hydrolysis of the glycosidic bond involves general acid catalysis. A carboxylate ion could be implicated in the active site of all three cellulase-system components. There is also evidence that the cellulose binding domain is highly glycosidated suggesting that the carbohydrate moiety may have an important role in their binding to cellulose.

Many models and hypotheses have been advanced to explain the mechanism of action of the cellulase-system in effecting the hydrolysis of cellulose. The original hypothesis put forward by Reese *et al.*, (1950) stated that crystalline cellulose was modified initially by an unknown component of cellulase (termed C_1) rendering the substrate swollen or amorphous, upon which the hydrolytic enzymes of cellulase (termed C_x) could then act. Over 45 years later, an amorphous or swollen cellulose-generating component of cellulase has still not been identified. The C_1 component was, however, designated as CBH and the C_x as EG activity. The factor C_1 could be the capacity or affinity of EG for adsorption on crystalline cellulose.

The classical action of cellulase is envisioned as an initial attack by endoglucanases (especially in the amorphous regions) followed by the combined action of cellobiohydrolases and endoglucanases, with final hydrolysis of the small oligosaccharides to glucose by ß-glucosidase. This model of sequential and cooperative attack provides a central foundation of the hydrolysis process. Fig. 2-5 shows a schematic representation of the synergistic relationship between the cellulase-system components in cellulose hydrolysis.

A widely accepted theory for the synergistic action between CBH and EG is the so-called 'endo-exo' model. Initially, EG hydrolyses internal ß-1,4-glcosidic bonds randomly in the chains at the surface of the cellulose fibers and thereby produce free chain ends. CBH then splits off cellobiose units and some glucose from the non-reducing ends of these chains one by one in an exo-like fashion. One problem with the 'endo-exo' model is that the underlying evidence is lacking, namely, that the enzymes called endoglucanases really act randomly and that cellobiohydrolases really split off cellobiose units from the non-reducing end of cellobiose chains. On the contrary, it has been shown that CBH I is able to hydrolyze both cellulose, cellodextrins and cellobiose into glucose at high enzyme concentration if incubated long enough.

Another problem is that synergism has been observed between enzymes which are both assumed to be exo-cellulases, e.g., CBH I and CBH II from *T. reesei.*

Each model cited has merit and perhaps each is at least transiently dominant during hydrolysis. EGs initiate attack (especially in amorphous regions) creating additional sites for hydrolysis by CBH to yield small oligosaccharides (mainly cellobiose). Furthermore, removal of cellobiose to form glucose by ß-glucosidase relieves its end-product inhibition towards EG and CBH. ß-glucosidase is analogously inhibited by its product glucose and here, relief of end-product inhibition is gained by subsequent oxidation via glucose or by glucose uptake by the fungus. ß-glucosidase has been shown to greatly increase the rate and extent of hydrolysis by ensuring the efficient hydrolysis of cellobiose and reducing the influence of end-product inhibition.

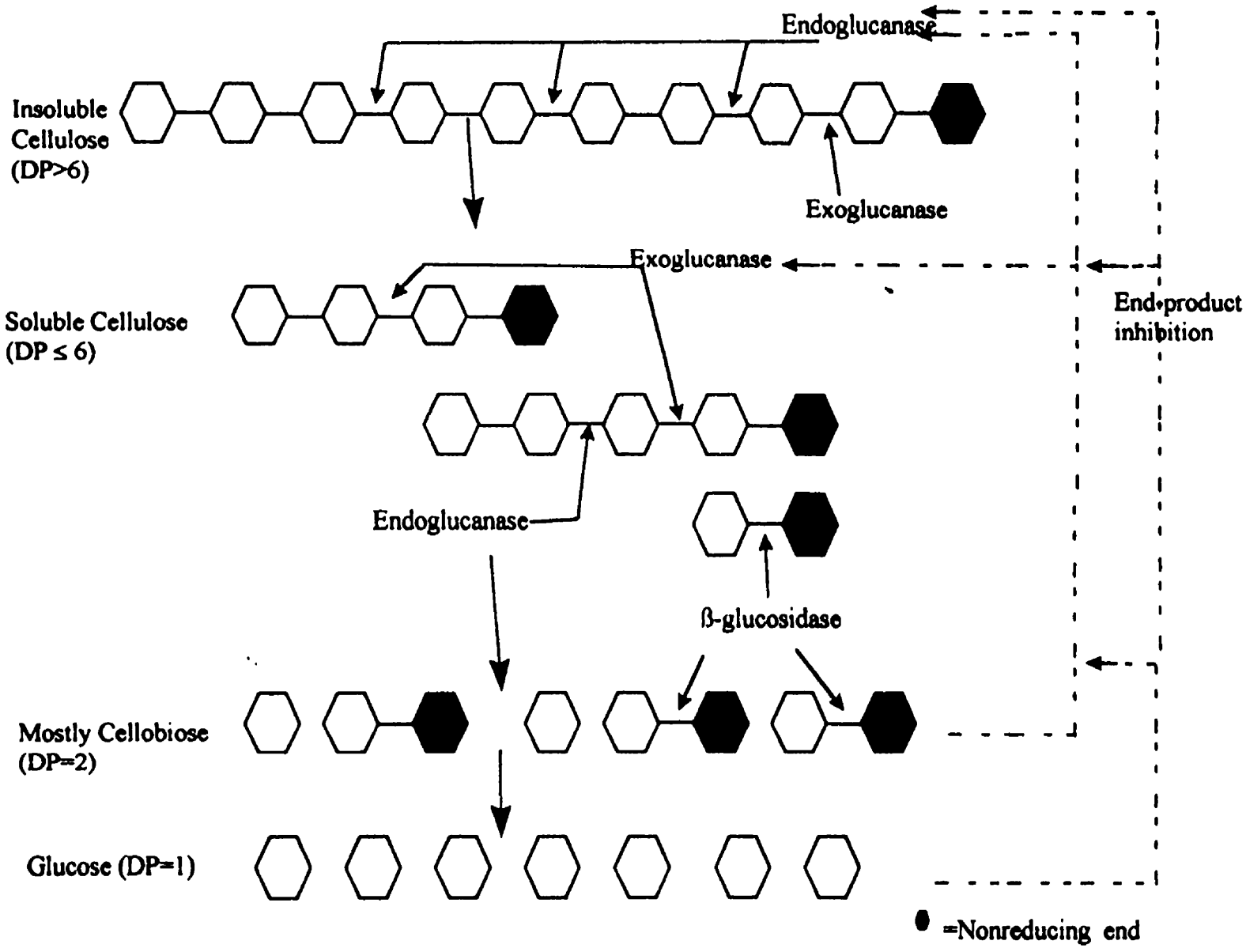


Fig.2-5: Schematic representation of the synergistic action of cellulase components on cellulose hydrolysis (Saddler, 1986)

2.9.1 Constraints in Cellulose Hydrolysis - A perspective

Lignocellulosic biomass can not be saccharified by enzymes to high yields without a pretreatment (Rao *et al.*, 1983). Various pretreatment techniques that have been used to enhance the susceptibility of the substrate to enzymes include mechanical (Tassinari *et al.*, 1982; Caulfield and Moore, 1974) and chemical (Saddler *et al.*,1982; Wilke *et al.*, 1981).

In the hydrolysis of natural lignocelluloses, lignin is an important obstacle for enzymatic digestion. Physically, lignin forms a barrier, suppressing the penetration of polysaccharide-digesting enzymes (Kirk and Haskin, 1973). Baker (1973) observed an inverse relationship between the lignin content and the digestibility of a wide variety of lignocelluloses. It has been reported by Gharpuray *et al.*, (1983), that the hydrolysis rate of wheat straw increased substantially with an increase in the extent of delignification, i.e., up to about 50% delignification. However, beyond this the hydrolysis rate increased only slightly. It has also been established that the less the lignin content of lignocelluloses, the higher the susceptibility to enzymatic hydrolysis and in vitro digestibility (Feist *et al.*, 1970).

Phenolic acids may be produced during certain pretreatments of lignocelluloses and these could also limit the accessibility of cellulose and hemicelluloses to enzymatic attack (Hartley, 1981).

Another obstacle in the hydrolysis of untreated lignocelluloses is the crystallinity of the cellulose component. Crystalline material which consists almost wholly of true cellulose is concentrated near the lumen and diminishes towards the primary wall. The cellulose

molecules are held together by very close packing and strong van der Waals forces of attraction. This makes the crystalline cellulose more resistant to enzymatic or acidic hydrolysis than that of the amorphous region (Sultze, 1957).

Furthermore, in the native state, the available area of gross capillaries (cell lumen, pits etc.) of lignocellulose cell walls are not enough to bring about considerable hydrolysis of cellulose because of the relatively larger cellulase molecules. Table 2-5, for instance, shows the porosity of cellulose biomass without pretreatment. It is estimated that there are about 600-800 m^2 of surface area per gram substrate, but approximately 98% of this area is in pores that are available only to molecules smaller than 5.1nm . Various studies have, however, shown that the cellulase molecule is about 5.1 nm. Cowling (1975) suggested that if cellulase is a sphere in aqueous solution, the diameter is about 5.0 nm and if it is ellipsoid, it is about 30 x 18.0 nm. Other studies by small-angle-X-ray scattering (SAXS) measurements have shown the cellulase molecule to be about 5.1 nm. An empirical size of 5.1 nm is used in much of the literature (Grethlein and Converse, 1991). It is, therefore, clear that if the approximate size of cellulase is 5.1 nm, then the enzyme is excluded from most of the surface area of substrates. The rate of hydrolysis is thus limited by the accessible surface area which mostly is the external bulk area of the cellulosic particle and the area of the internal lumina (Cowling, 1975).

Table 2-5: Calculated Surface Area from Solute Exclusion Measurements for Various Woods (m^2/g)

Substrate	Area available to various sized molecules		
	0.4 nm	5.1 nm	9.0 nm
Mixed hardwood	846	10.5	2.0
Poplar	645	6.5	4.0
White pine	800	13.7	5.8
Steam extracted pine	745	13.9	2.6

Source: Grethlein (1985)

The polysaccharides in lignocellulosic materials can also be converted to sugars by acid hydrolysis, using either dilute or concentrated acid namely H_2S0_4, HCl and hydrofluoric acid (Vallander and Eriksson, 1984). However, there are corrosion problems particularly with HCl and H_2S0_4 resulting in increased cost, low yields as well as potentially adverse environmental effects due to emissions from the acid hydrolysis processes. On the other hand, enzymes are acceptable environmentally but expensive to produce (Perez *et al.*, 1980 ; Esterbauer *et al.*, 1991).

2.10 Relationship Between Lignocellulosic Pretreatments, Cellulase-System Production, and Cellulose Hydrolysis

Lignocellulose like wheat straw is a recalcitrant heterogeneous material (Fig. 2-2) and pretreatment is a requisite before it can be used for either cellulase-system production or for its hydrolysis into monomeric sugars which can be subsequently fermented into ethanol.

The choice of pretreatment will predicate the type of end use of the substrate since in general, mild pretreatments do not render the substrate susceptible enough for hydrolysis while on the other hand harsh pretreatments suitable for cellulose hydrolysis are not suitable for cellulase-system production. This is because harsh pretreatments are more likely to produce inhibitory compounds which do not auger well for the growth of cellulolytic microorganisms (Gharpuray *et al.*, 1983). Gharpuray *et al.*, (1983) even concluded that optimum hydrolysis of wheat straw requires pretreatment of 0.1g NaOH per g substrate.

An effective pretreatment of lignocelluloses for cellulase-system production should expose and increase the surface area of available cellulose and hemicelluloses for their access to the cellulolytic organism. It should also be able to mildly alter the cellulose structure for the cellulose to act as an efficient carbon source. An effective pretreatment for cellulose hydrolysis on the other hand should in addition to the above, be able to increase the surface area to pore size ratio for the cellulase enzyme to be able to pass through and still remain in physical contact with cellulose. This orientation is important to promote extensive hydrolysis of the substrate.

Several reviews have covered pretreatment methods that enhance cellulose hydrolysis (Millet *et al.*, 1975 ; Chang *et al.*, 1981 ; Fan *et al.*, 1981 ; Weil *et al.*, 1994) but pay little attention to pretreatment methods that enhance lignocelluloses for cellulase-system production.

Surfactants such as Tween 80, polyethylene glycol (PEG) and certain biosurfactants (sophorolipid, rhamnolipid) play an important role in the expression of cellulases during extraction and in the prevention of cellulase-system inactivation. Surfactants are amphiphilic molecules which turn to adsorb onto surfaces and provide a means of altering surface and interfacial properties of the reaction system. They lower the surface tension or the free energy between a solution and the gas phase in equilibrium with it. Several reports have shown that surfactants are capable of preventing cellulase inactivation (Tjerneld *et al.*, 1991; Jones and Lee, 1988 ; Reese, 1980) possibly by interfering with the hydrophobic portion of the cellulase. Enzyme inactivation is characterized by protein unfolding followed by aggregation due in part to hydrophobic interactions which are weakened in the presence of nonionic detergents.

2.11 Lignocellulose Conversion Processes for Ethanol Production

There are basically five steps involved when lignocelluloses are considered for ethanol production (Fig. 2-6):

i. Pretreatment of the lignocellulose to make available the cellulose and hemicelluloses components for either the production of cellulase-systems or for their enzymatic hydrolysis

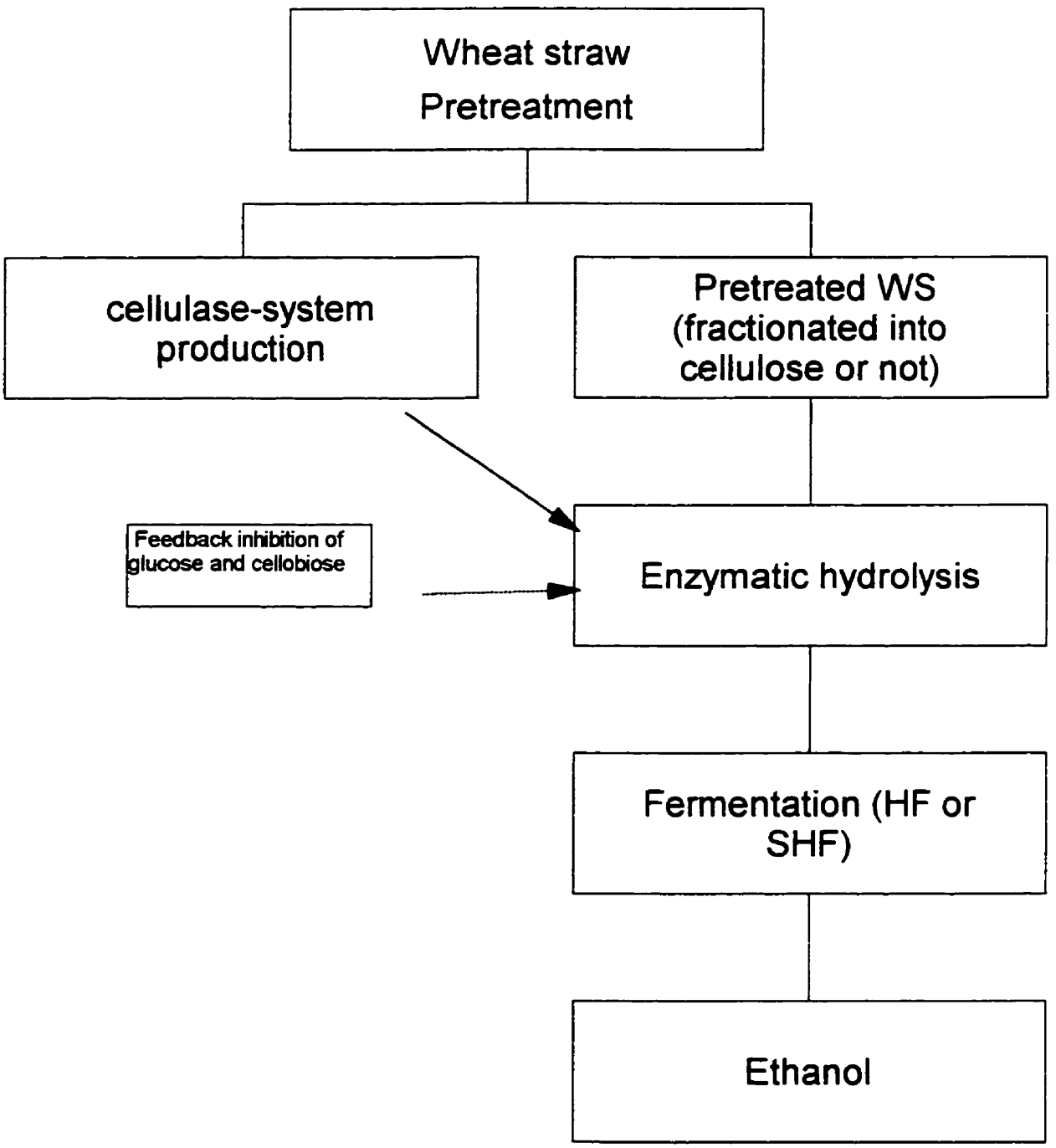
ii. The production of a complete cellulase-system from mild pretreated lignocelluloses, the purpose of which is to attain high filter paper activity and ß-glucosidase activity with the ratio of the former to the latter close to one. This is important to alleviate end-product inhibition and attain complete cellulose hydrolysis

iii. Enzymatic hydrolysis of pretreated lignocelluloses or fractionated cellulose thereof to obtain hexose and pentose sugars as fermenting materials

iv. Fermentation of the soluble sugars with suitable yeasts either by separate processes of hydrolysis and fermentation (HF), or by simultaneous hydrolysis and fermentation (SHF) where the suagrs serve as an energy source for growth and maintenance and generate alcohol as a waste product

v. Distillation of the alcohol from the fermentation broth.

Fig. 2-6 Conversion of wheat straw (WS) into fuel ethanol



2.11.1 Separate Hydrolysis and Fermentation (HF), and Simultaneous Hydrolysis and Fermentation (SHF) - An Overview

In a separate hydrolysis and fermentation process, the substrate is enzymatically hydrolyzed separately and the resulting sugars, separated or not from unutilized substrate, are fermented with suitable yeast for ethanol production. On the other hand, a simultaneous hydrolysis and fermentation process involves ethanol production with the enzymatic hydrolysis and yeast fermentation processes occurring simultaneously.

Both approaches have their inherent advantages and disadvantages but SHF appears to have a slight edge over HF because of the following reasons:

i. End-product inhibition is minimized since sugars are fermented as they are released by hydrolysis. It is estimated that approximately 1 mM glucose is enough to inhibit ß-glucosidase (Ooshima *et al.*, 1985), while glucose and cellobiose concentrations of about 1% could inhibit cellobiohydrolases and endoglucanases of the *Trichoderma* cellulase-system (Maguire, 1977; Holzapple *et al.*, 1990; Fujii *et al.*, 1991)

ii. Less time is required to complete the ethanol production process with less energy input, thus, high productivity

iii. The continuous presence of ethanol with low sugars because of their fast uptake could result in reducing microbial contamination in the process. Even routine separate hydrolysis experiments require antibiotics or other antimicrobial agents to combat microbial contamination (Reese and Mandels, 1980)

Notwithstanding the advantages mentioned for SHF, HF also has its advantages, notably, that it can accommodate high temperatures (40-50°C) for enzymatic hydrolysis and lower

temperatures (30-37°C) for optimum fermentation by yeasts. This paradox could be overcome in SHF with thermophilic microorganisms which still remain elusive.

CHAPTER 3

PRODUCTION OF CELLULASE-SYSTEMS OF *TRICHODERMA REESEI*: THEIR COMPOSITION AND GROWTH CHARACTERISTICS UNDER SOLID - STATE FERMENTATION AND LIQUID- STATE FERMENTATION SYSTEMS.

3.0 CONNECTING STATEMENT

This comparative study was conducted to delineate the two approaches for cellulase-system production and to provide information on cultural parameters specific to wheat straw for subsequent optimization studies.

Note : This chapter constitutes text of a paper submitted for publication as follows :

Awafo, V.A., Chahal, D.S and Simpson, B.K. 1997. Production of cellulase-systems of *Trichoderma reesei*: Their composition and growth characteristics under solid-state fermentation and liquid-state fermentation systems. J. Food Biotechnol.

Contributions of Co-authors : Chahal, D.S. and Simpson, B.K (Thesis co-supervisors)

3.1 ABSTRACT

The potential for the commercialization of cellulose hydrolysis into fermentable sugars for ethanol production is largely hindered by the cost of cellulases and, therefore, requires critical examination of environmental parameters as they relate to the cellulase-system production from specific substrates. The cell bound ß-glucosidase is much better quantitatively expressed with the onset of autolysis and was much more pronounced in solid-state fermentation (SSF) cultures than in liquid state fermentation (LSF) cultures. *T. reesei* mutants QMY-1 and MCG 80 also produced higher filter paper activity (FPA) from SSF cultures than from LSF cultures. The consequence of higher ratios of ßGA to FPA from SSF than from LSF resulted in higher cellulose hydrolysis to produce more reducing sugars.

3.2 INTRODUCTION

There are two well known methods by which cellulase-systems can be produced; liquid-state fermentation (LSF) and solid-state fermentation (SSF). In SSF, insoluble substrate is fermented with enough moisture without free water while in LSF, the substrate is solubilized or suspended as fine particles in a large volume of water (Chahal, 1985). The end objective in producing cellulase-systems is to attain good quality enzyme for a viable process of cellulose hydrolysis into fermentable sugars since cellulase alone is estimated to be over 50% the total cost of producing glucose from cellulose (Hendy *et al.*, 1984). There are, however, several approaches to reduce enzyme cost: production of high quality

cellulase-systems with high filter paper activity and ratio of ß-glucosidase activity to FPA close to one, and that is also capable of being recycled in the hydrolytic step, effective pretreatment of lignocelluloses; optimization of fermentation parameters, and using cheaper fermentation medium components. The mode of fermentation and type of microorganism are imperative in the evaluation of any approach aimed at reducing the cost of cellulase-systems.

The cellulase-system of *Trichoderma reesei* has been widely cultivated in LSF systems under batch, fed batch and continuous conditions (Shoemaker *et al.*, 1981; Doppelbauer *et al.*, 1987; Allen and Andreotti, 1982) and to a limited extent in SSF systems (Toyama, 1976; Chahal, 1985) with several improved *T. reesei* strains from the parental strain QM6a. However, the literature contains conflicting cellulase activity values from most of these strains on similar substrates. This may be attributable to differences in substrate pretreatments, culture media composition, fermentation equipment, reference samples and analytical procedures. This study aims at comparing the composition of the cellulase-systems of *T. reesei* mutants from wheat straw under both LSF and SSF conditions as a prelude to selecting one or two for subsequent optimization studies.

3.3 MATERIALS AND METHODS

3.3.1 Substrate and Pretreatment

Wheat straw (WS) samples were obtained from the Macdonald Campus farm of McGill University, Montreal. They were ground in a Wiley mill with mesh screen of 20 and the

moisture content was determined as adapted from the Association of Official Analytical Chemists (A.O.A.C, 1984). Sodium hydroxide was added at 0.05 g/g and 0.10 g/g dry weight of WS and distilled water was added to bring the final moisture content to 70%. The samples were thoroughly mixed and left overnight at room temperature for the NaOH to be imbibed by the substrate particles. They were further autoclaved at 121°C at 80 Psig for 1 h.

3.3.2 Microorganisms and Culture Medium

Trichoderma reesei QMY-1 (NRRL 18760) and MCG 80 (NRRL 12368) were maintained at 4°C on Mandels medium (Mandels and Weber, 1969) with delignified wheat straw as carbon source. *T. reesei* QMY-1 was obtained from D.S. Chahal, Institut Armand-Frappier, Laval, Canada while *T. reesei* MCG 80 was supplied by J.L. Swezey, ARS Patent Culture Collection, USDA, Illinois, USA.

Mandels standard medium with some modifications was used for the cultivation of the microorganisms. The composition of the modified medium was calculated based on the carbohydrate content of WS (Staniforth, 1979) and the amount of water added brought the final medium composition to 100mL/4 g WS for the LSF cultures and 4mL/4 g WS for the SSF cultures.

The composition of the modified Mandels medium for one gram of WS per 100 mL for LSF cultures and per 4 mL for SSF cultures was: KH_2PO_4, 0.56 g; $(NH_4)_2SO_4$, 0.392 g; $CaCl_2$, 0.084 g; $MgSO_4.7H_2O$, 0.084 g; yeast extract, 0.14 g; $CoCl_2$, 0.56 mg;

$ZnSO_4.7H_2O$, 0.392 mg; $MnSO_4.H_2O$, 0.448 mg; $FeSO_4.7H_2O$, 1.4 mg; Tween 80, 0.056 mL and 4 g WS.

3.3.3 Fermentation and Cellulase-System Production

The WS samples containing the fermentation medium were adjusted to pH 6 and 5 and autoclaved at 121°C for 30 min. The samples were aseptically inoculated with *T. reesei* mutants grown on Mandels medium for 36 h from 72 h old pre-inoculums. The rest of the experimental set up were as detailed below. The SSF samples were held in humidified incubators (Norco 4100), while the LSF samples were placed in incubator shakers (New Brunswick Scientific, N.J) with agitation controlled at 200 rpm.

3.3.3.1 SSF Set up- Effect of Temperature Shift and pH on Cellulase-System Activities

Temperature (30°C and/or 25°C) and pH (pH 5 and 6) were used to determine their effect on the growth characteristics and activity of cellulase-systems. The experimental set up is given in Table 3-1. Each experiment was run in duplicate.

Table 3-1: Experimental Parameters used for SSF

Run no.	Temperature (°C)	pH	Duration of SSF (weeks)
1	30	6	3
2	30	5	3
3	25	6	3
4	25	5	3
5	30 to 25	6	1 week at 30°C, then 2 weeks at 25°C
6	30 to 25	5	1 week at 30°C, then 2 weeks at 25°C
7	25 to 30	6	1 week at 25°C, then 2 weeks at 30°C
8	25 to 30	5	1 week at 25°C, then 2 weeks at 30°C

3.3.3.2 LSF set up- Effect of Temperature and pH on Cellulase-System Activities

The LSF samples were subjected to either 30°C or 25°C incubation temperatures and the pH maintained at either 6 or 5 for 7 days with either 10% NaOH or 10% H_2SO_4.

3.3.4 Effect of Incubation on the Activity of Cellulase-Systems in SSF and LSF

It is important to establish the optimum period for the harvest of cellulase-systems in both LSF and SSF so as to maximize their activities and to reduce cost in production. Wheat straw samples subjected to NaOH pretreatment at 5% (0.05 g NaOH/g substrate) were

used to study the effect of incubation time at 30°C, pH 6 for the *T. reesei* mutants under both SSF and LSF conditions

3.3.5 Enzyme Extraction and Analysis

The SSF samples were extracted in a total volume of 100 mL distilled water by shaking in incubator shakers for 30 min at 200 rpm and centrifugation at 11 000 g for 30 min. The LSF samples, on the other hand, were subjected to centrifugation only at 11 000 g for 30 min without shaking.

The extracted samples were analyzed for filter paper activity (FPA) and ß-glucosidase activity (ßGA) by the modified method of Mandels *et al.*, (1976) as approved by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987). FPA is the amount of international units (IU) of glucose (µmol /min) released from 50 mg microcrystalline filter paper (Whatman chromatography paper) after 60 min incubation at 50°C while ßGA was expressed in IU using 0.5% salicin solution after 30 min incubation at 50°C. The ratio of the cellulase activity was obtained by dividing ßGA by FPA.

3.3.5 Evaluation of the Quality of Cellulase-Systems from SSF and LSF Cultures

Cellulase-systems from both SSF and LSF were selected for the hydrolysis of 5% delignified wheat straw and Avicel PH-101 (Fluka chemika) based solely on the ratio of ß-glucosidase activity to filter paper activity. The hydrolysate components were quantitatively determined by HPLC analysis (Awafo *et al.*, 1996)

3.4 RESULTS AND DISCUSSION

3.4.1 Relationship Between Cultural Parameters, Visible Growth and Cellulase-System Production

Tables 3-2a ,3-2b and 3-3 illustrate the following :

i. When wheat straw was pretreated with NaOH at concentrations as high as 10 % w/w (0.1 g NaOH/g substrate) no visible growth was observed. This was reflected in the low cellulase-system activity values suggesting that high concentration of NaOH (0.1 g NaOH/g substrate) optimum for significant increases in the rate and extent of hydrolysis of lignocelluloses as reported by Choudhurry *et al.*, (1984) and Gray *et al.*, (1978) does not support the growth of some cellulase-system *T. reesei* mutants and hence cellulase-system production. This is significant and illustrates that only half the concentration optimum for cellulose hydrolysis is required for cellulase-system production (Table 3-2b). This is particularly helpful in optimization studies for cellulase-system production.

ii. Shift in temperature between 25°C and 30°C neither affects the growth of *T. reesei* mutants QMY-1 and MCG 80 nor the activity of their cellulase-systems. It was, however, observed that visible growth appeared first with samples incubated at 30°C.

iii. Both *T. reesei* mutants QMY-1 and MCG 80 were capable of growing and producing cellulase-systems with pH 7 and 5. However, *T. reesei* MCG 80 showed preference for the higher pH and *T. reesei* QMY-1 for low pH.

iv. The direct relationship between good and profuse growth of *T. reesei* mutants on pretreated substrate and cellulase-system was not established except that visible growth was an excellent indicator of the potential for cellulase-system production.

v. The cellulase-system activities (ßGA and FPA) were lower for the LSF cultures than those obtained from the SSF (Tables 3-2b and 3-3)

3.4.2 The Effect of Incubation on Cellulase-System Production under SSF and LSF Conditions

The incubation period has the inherent ability to affect both the quality and quantity of cellulase-systems produced because of autolysis. ß-glucosidase which is cell bound can only be expressed quantitatively with the onset of autolysis. The onset of autolysis has two effects on cellulase-system production. It enables the release of ß-glucosidase into the fermentation medium for easy extraction but may also affect the specific activity of the cellulase-system produced. Prolonged fermentation in the absence of any sustainable energy increases the tendency of proteolytic enzymes excreted extracellularly to act on available protein and start a degradation process aimed at providing the organism with sustainable energy. Autolysis of older mycelium is a common phenomenon in fungi (Cochraine, 1958). Figure 3-1 shows that for both mutants of *T. reesei*, the optimum cellulase activity was obtained after 3 weeks of incubation and that the low values of ß-glucosidase during the first two weeks may be as a result of the fact that during this period the cells were still in active growth and the cell bound ß-glucosidase could not be expressed extracellularly. One other observed phenomenon was that FPA appeared to tail

off after 3 weeks while ß-glucosidase activity increased slightly up to 4 weeks supporting the fact that there was onset of autolysis after 3 weeks of fermentation.

Figures 3-1 and 3-2 show the profiles for the production of cellulase-systems in SSF and LSF cultures with *T. reesei* QMY-1 and MCG 80. The filter paper activities (FPA) and ß-glucosidase activities (ßGA) were lower for LSF than those obtained under the SSF conditions. For instance, after 22 days of fermentation the FPA and ßGA for *T. reesei* QMY-1 under LSF were 3.23 IU/mL and 1.16 IU/mL respectively, whereas after 21 days of fermentation under SSF conditions, the FPA and ßGA were respectively, 6.51 IU/mL and 6.24 IU/mL.

3.4.4 Overall Advantages of SSF Over LSF in the Production of Cellulase-Systems of *T. reesei*

i. SSF cultures can be extracted to yield high cellulase-system activity values by centrifugation and adjusting the volume of extraction liquid, whereas to obtain high values of the cellulase-system activities from LSF cultures will require more complex separation techniques

ii. SSF cultures require no agitation during fermentation and can be fermented for longer periods of time with less chances of contamination than LSF cultures

iii. The substrate concentration in LSF cultures is severely limited because of problems of agitation and aeration compared to SSF cultures

iv. A combination of high substrate concentration and low volume of extraction liquid will produce higher cellulase-system activity values in SSF than could be obtained from LSF cultures

v. The lower ß-glucosidase activity value from LSF cultures means the ratio of ßGA to FPA for LSF cultures is very low with plausible effects on cellulose hydrolysis since lower ratios could result in the accumulation of cellobiose during cellulose hydrolysis.

3.4.5 Comparative Assessment of the Hydrolytic Potential of Cellulase-Systems Obtained from SSF and LSF Cultures

The results demonstrated that lower ratios of ß-glucosidase to FPA were obtained from LSF cultures and vice versa for SSF cultures. These cultures from the different cellulase

sources with different ratios of ßGA: FPA were used to establish the extent to which these ratios affect cellulose hydrolysis.

Figures 3-3 and 3-4 show that reducing sugars obtained from cellulose hydrolysis were affected by the ratio of ßGA to FPA. Both delignified wheat straw and avicel showed similar trends in the production of reducing sugars with the former producing more than the latter. There was an over all increase in glucose yield and decrease in cellobiose content with increase in the ratio of ßGA: FPA. Xylose and arabinose were apparently not affected by the ratio content of ßGA: FPA. The significance of the ratio of ßGA to FPA has been examined by some researchers (Breuil *et al.*, 1992 ; *Stockton et al.*, 1991). Both studies were, however, based on either pure celluloses or commercial enzymes thus limiting their significance for potential commercial applications with lignocelluloses.

3.5 CONCLUSION

Solid-state fermentation has a comparative advantage over liquid-state fermentation in the production of cellulase-systems from *T. reesei*. Liquid state fermentation produced less ß-glucosidase than solid-state fermentation which resulted in lower hydrolysis products from the former. The parameters for cellulase-system production, however, need to be optimized to attain maximum levels of their activity and hydrolytic potentials.

Table 3-2a Effect of NaOH concentration used for the pretreatment, initial pH and shift in temperature for growth, on the production of cellulase-systems from *T. reesei* mutants under solid-state fermentation conditions.

Run No.	Incubation temperature (°C)	*T. reesei* mutant	NaOH (% w/w)	pH	Cellulase-system activity		
					FPA		ßGA
					IU/mL	IU/g	
1	30	MCG 80	10	7	0.58	14.5	ND
2	30	MCG 80	10	5	0.29	7.2	ND
3	30	QMY-1	10	7	0.29	7.2	ND
4	30	QMY-1	10	5	0.24	6.0	ND
5	25	MCG 80	10	7	0.59	14.7	ND
6	25	MCG 80	10	5	0.32	8.0	ND
7	25	QMY-1	10	7	0.27	6.7	ND
8	25	QMY-1	10	5	0.23	5.7	ND
9	30 ⇒ 25	MCG 80	10	7	0.59	14.7	ND
10	30 ⇒ 25	MCG 80	10	5	0.30	7.5	ND
11	30 ⇒ 25	QMY-1	10	7	0.24	6.0	ND
12	30 ⇒ 25	QMY-1	10	5	0.27	6.7	ND
13	25 ⇒ 30	MCG 80	10	7	0.49	12.2	ND
14	25 ⇒ 30	MCG 80	10	5	0.33	8.2	ND
15	25 ⇒ 30	QMY-1	10	7	0.28	7.0	ND
16	25 ⇒ 30	QMY-1	10	5	0.34	8.5	ND

The cellulase-system activity values are mean values of two determinations. There was no significant difference between the environmental parameters of pH and temperature and *T. reesei* mutant in cellulase-system production ($p \leq 0.05$)

Table 3-2b Effect of NaOH concentration used for the pretreatment, initial pH and shift in temperature for growth, on the production of cellulase-systems from *T. reesei* mutants under solid-state fermentation conditions.

Run No.	Incubation temperature (°C)	*T. reesei* mutant	NaOH (% w/w)	pH	Cellulase-system activity			
					FPA		ßGA	
					IU/mL	IU/g	IU/mL	IU/g
17	30	MCG 80	5	7	6.5	162	5.5	137
18	30	MCG 80	5	5	4.8	120	5.0	125
19	30	QMY-1	5	7	5.0	125	4.3	107
20	30	QMY-1	5	5	5.4	135	3.5	87
21	25	MCG 80	5	7	5.8	145	4.5	112
22	25	MCG 80	5	5	4.5	112	3.1	77
23	25	QMY-1	5	7	4.8	120	2.9	72
24	25	QMY-1	5	5	5.0	125	3.5	87
25	30 ⇒ 25	MCG 80	5	7	6.0	150	5.3	132
26	30 ⇒ 25	MCG 80	5	5	4.5	112	3.8	95
27	30 ⇒ 25	QMY-1	5	7	4.0	100	3.2	80
28	30 ⇒ 25	QMY-1	5	5	5.3	132	2.9	72
29	25 ⇒ 30	MCG 80	5	7	5.8	145	3.7	92
30	25 ⇒ 30	MCG 80	5	5	5.5	137	4.5	112
31	25 ⇒ 30	QMY-1	5	7	3.9	97	2.1	52
32	25 ⇒ 30	QMY-1	5	5	4.8	120	3.6	90

The cellulase-system activity values are mean values of two determinations. There was no significant difference between the environmental parameters of pH and temperature and *T. reesei* mutant in cellulase-system production ($p \leq 0.05$)

Table 3-3 Effect of NaOH concentration used for the pretreatment, initial pH and temperature for growth, on the production of cellulase-systems from *T. reesei* mutants under liquid-state fermentation conditions.

Run No.	Incubation temperature (°C)	*T. reesei* mutant	NaOH (% w/w)	pH	Cellulase-system activity			
					FPA		ßGA	
					IU/mL	IU/g	IU/mL	IU/g
1	30	MCG 80	5	7	3.4	85	1.6	40
2	30	MCG 80	5	5	2.2	55	0.6	15
3	30	QMY-1	5	7	1.6	40	0.5	12
4	30	QMY-1	5	5	1.3	32	0.3	7
5	25	MCG 80	5	7	2.9	72	1.1	27
6	25	MCG 80	5	5	3.1	77	0.9	22
7	25	QMY-1	5	7	1.8	45	0.4	10
8	25	QMY-1	5	5	1.4	35	0.5	12
9	30 ⇒ 25	MCG 80	5	7	1.2	30	0.7	17
10	30 ⇒ 25	MCG 80	5	5	0.9	22	0.2	5
11	30 ⇒ 25	QMY-1	5	7	1.4	35	0.4	10
12	30 ⇒ 25	QMY-1	5	5	2.5	62	0.6	15
13	25 ⇒ 30	MCG 80	5	7	3.5	87	1.3	32
14	25 ⇒ 30	MCG 80	5	5	2.9	72	0.8	20
15	25 ⇒ 30	QMY-1	5	7	2.2	55	0.8	20
16	25 ⇒ 30	QMY-1	5	5	2.5	62	0.6	15

The cellulase-system activity values are mean values of two determinations. There was no significant difference between the environmental parameters of pH and temperature and *T. reesei* mutant in cellulase-system production ($p \leq 0.05$)

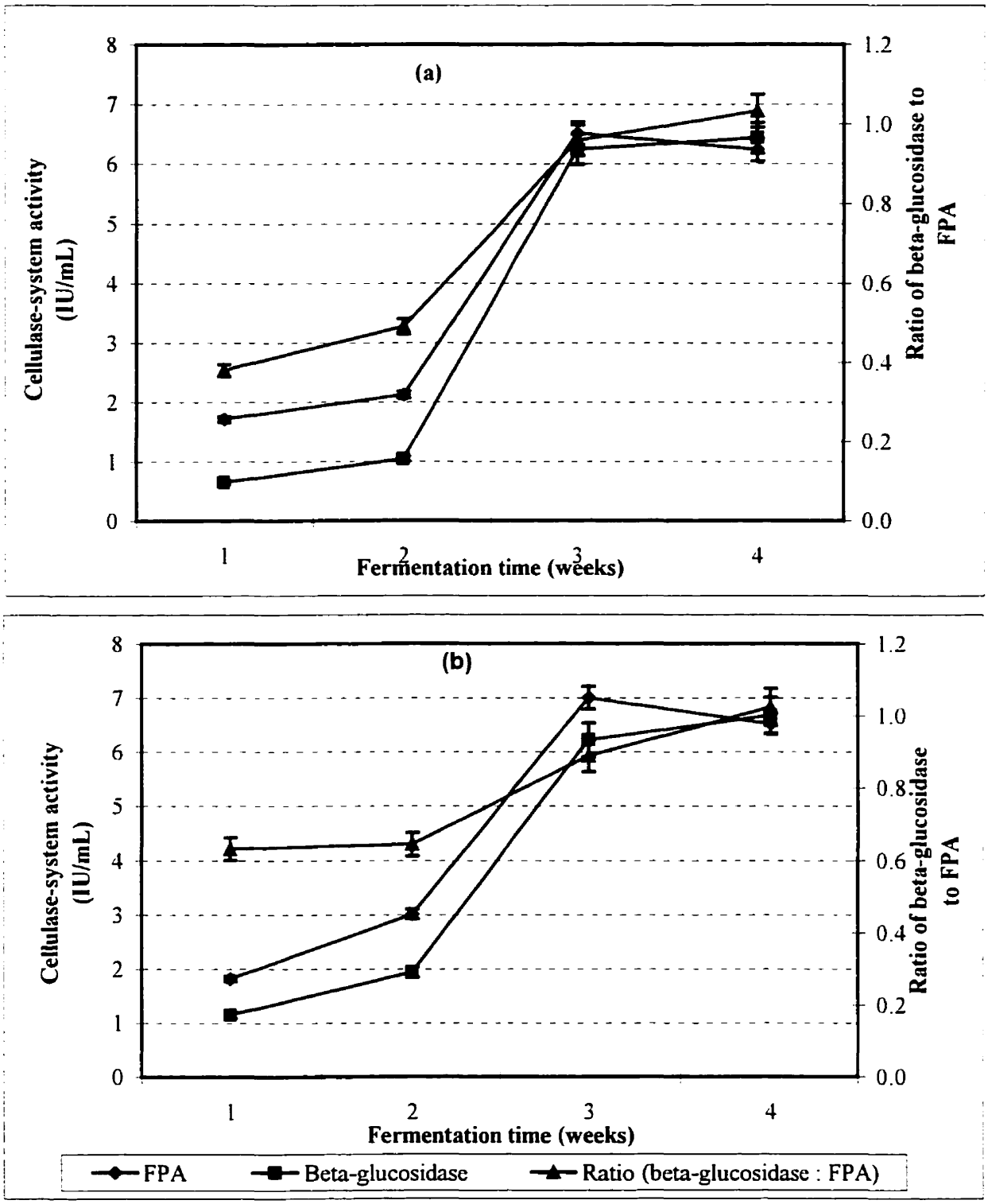


Fig. 3-1 Effect of harvesting time on the activity of the cellulase-system of T. reesei mutant (a) QMY-1 and (b) MCG 80 under SSF conditions

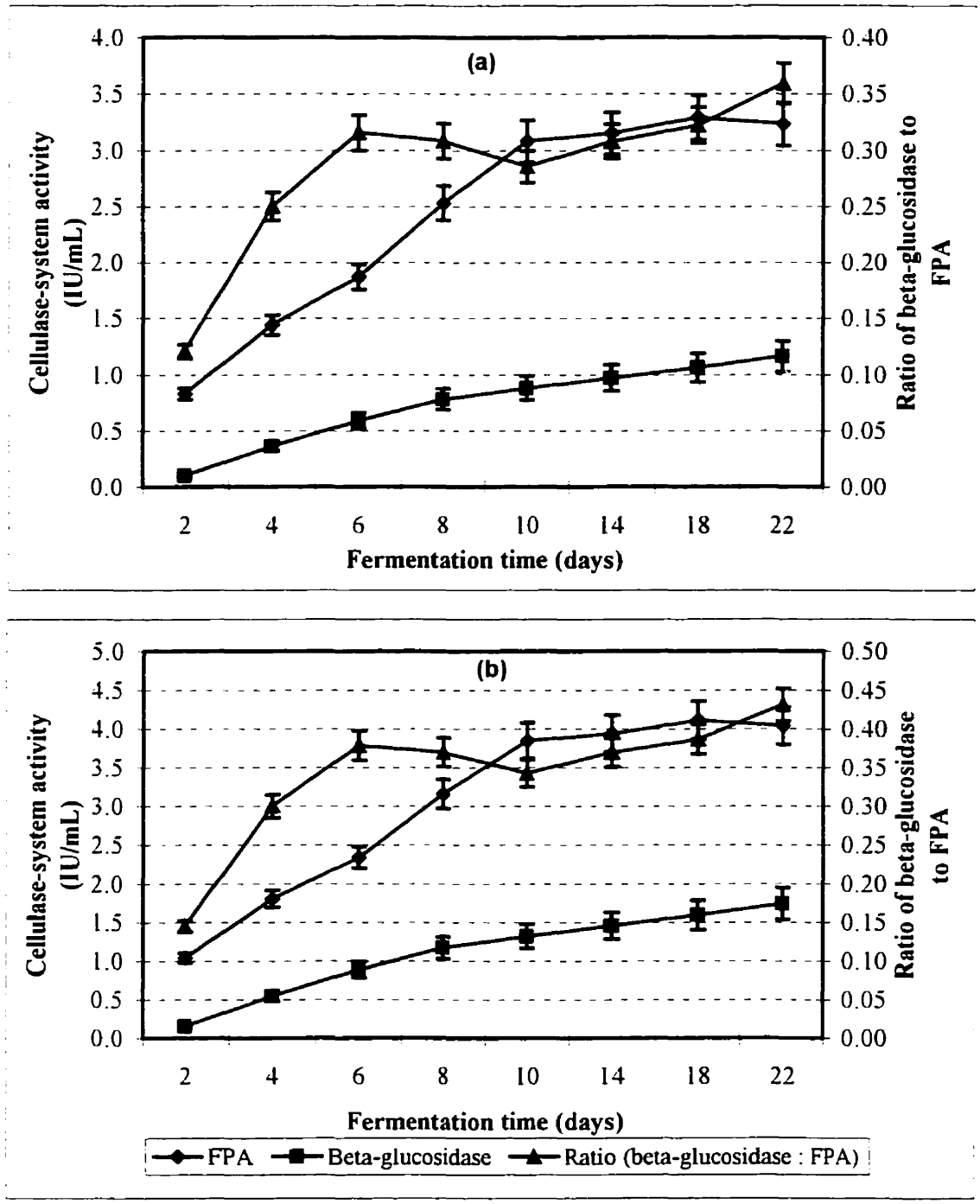


Fig. 3-2 Effect of harvesting time on the activity of the cellulase-system of T. reesei mutant (a) QMY-1 and (b) MCG 80 under LSF conditions

Fig 3-3 Effect of the ratio of beta-glucosidase activity to filter paper activity on the hydrolysis of delignified wheat straw

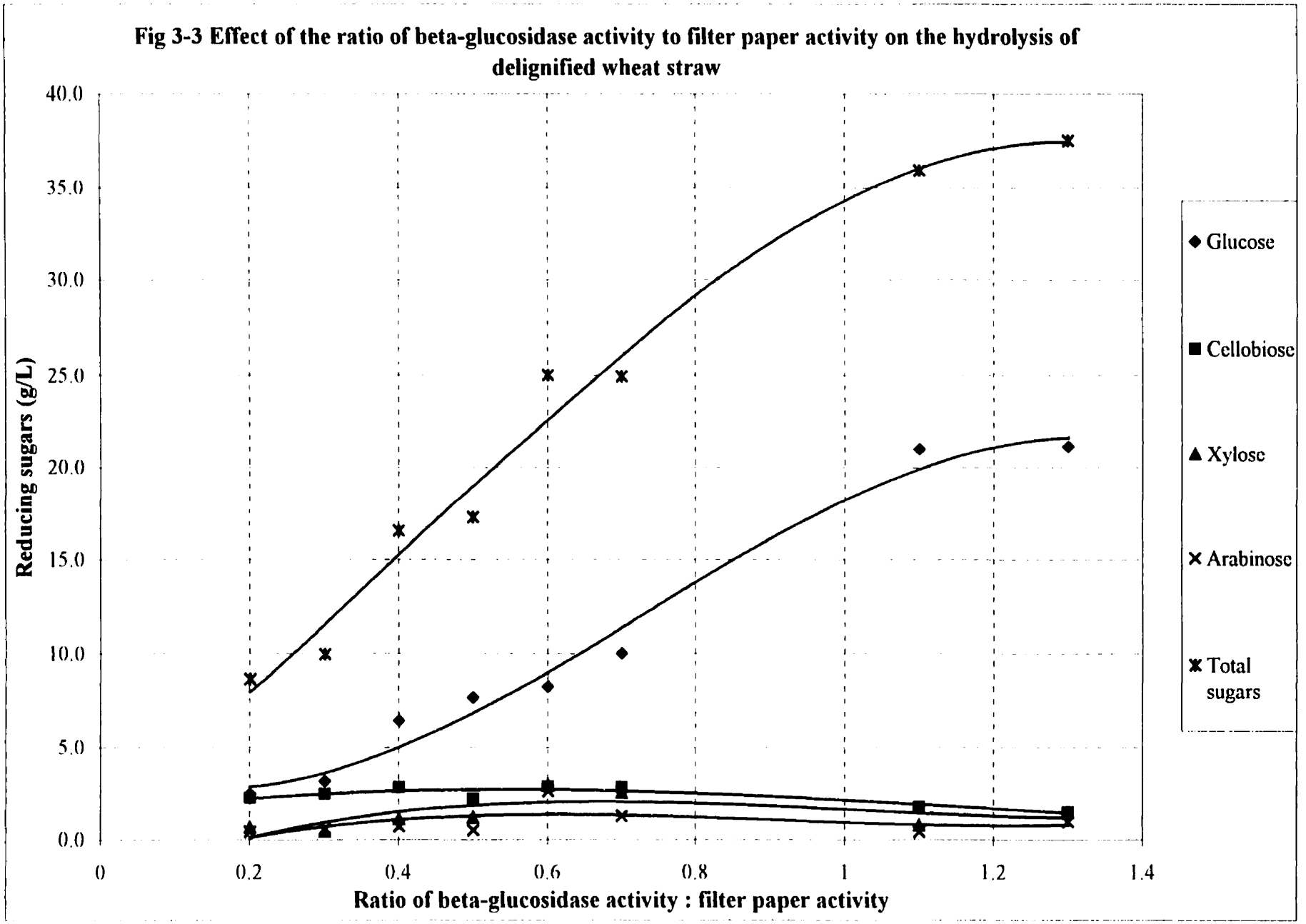
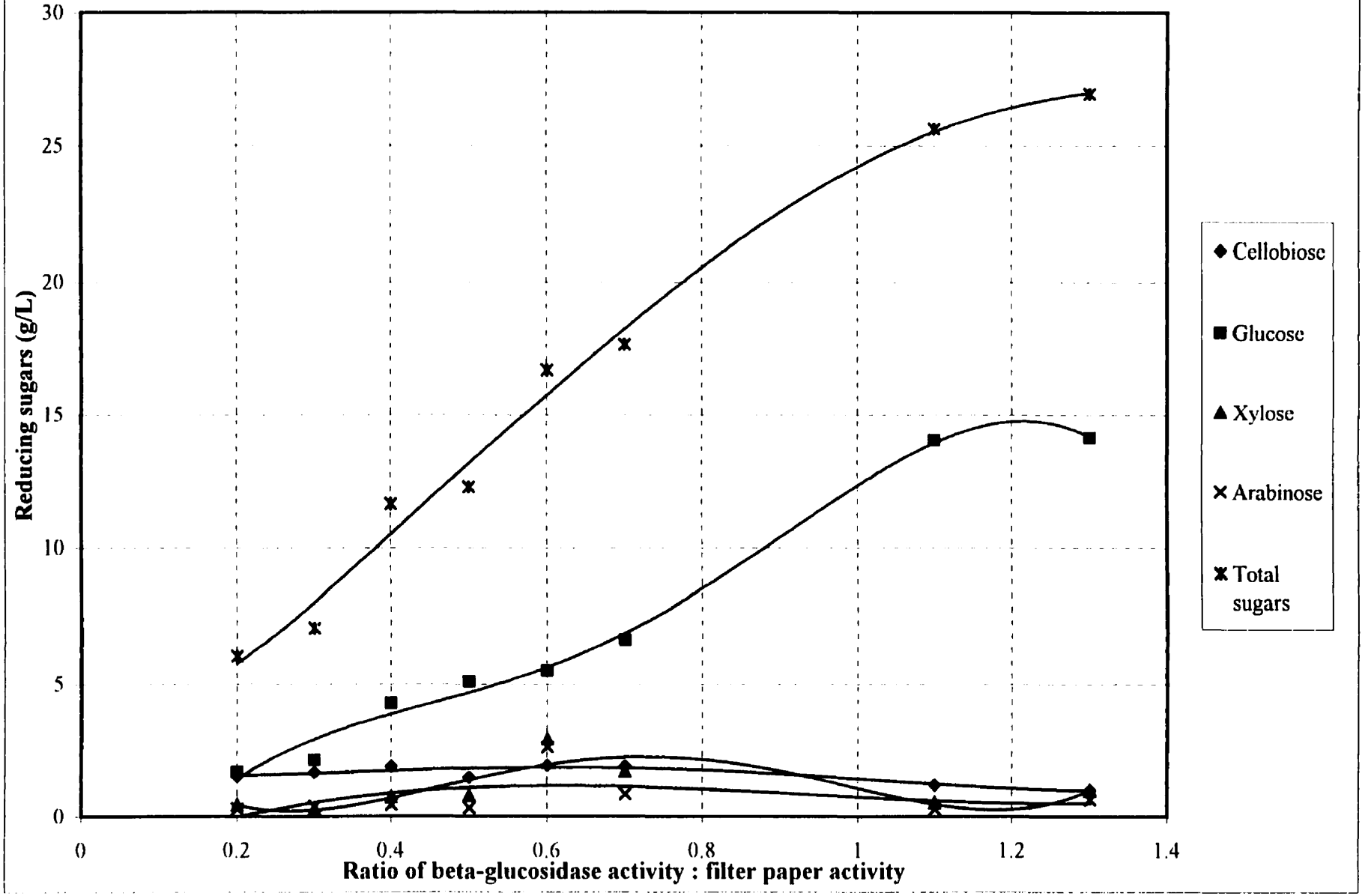


Fig. 3-4 Effect of the ratio of beta-glucosidase activity to filter paper activity on the hydrolysis of avicel



CHAPTER 4

COMPARATIVE EVALUATION OF MILD SODIUM HYDROXIDE AND ITS COMBINATION WITH STEAM EXPLOSION AS PRETREATMENTS OF WHEAT STRAW FOR THE PRODUCTION OF CELLULASE-SYSTEMS OF TWO *T. REESEI* MUTANTS UNDER SOLID-STATE FERMENTATION CONDITIONS.

4.0 Connecting Statement

In the previous chapter, the substrate pretreatment level and cultural conditions were selected from the literature. As a result of the higher cellulase-system activity results obtained from SSF over LSF, this chapter considers optimization models to optimize pretreatment and cultural conditions in order to establish their optimum levels for maximum cellulase-system production. The hydrolytic potential of the cellulase-systems were also evaluated to underscore the relevance of different cellulase-system sources. It may be remarked that the economics of cellulase production has in the past been based on cellulase activity levels obtained from LSF systems.

Note: This chapter constitutes the text of a paper submitted for publication as follows:

Awafo, V.A., Chahal, D.S. and Simpson, B.K. 1997. Comparative Evaluation of Mild Sodium Hydroxide and its Combination with Steam Explosion as Pretreatments of Wheat Straw for the Production of Cellulase-Systems of Two *T. reesei* Mutants Under Solid-state Fermentation Conditions. Bioresource Biotechnol.

Contribution of Co-authors: Chahal, D.S. and Simpson, B.K. (Thesis co-supervisors)

4.1 ABSTRACT

A central composite orthogonal design model was used to optimize pretreatment and cultural conditions for the production of cellulase-systems of *T. reesei* QMY-1 and MCG 80 from wheat straw. The model was capable of predicting conditions for maximum filter paper activity (FPA) and ß-glucosidase activity (ßGA). Statistical analysis showed close agreement between the experimental cellulase-system activities (FPA and ßGA) and predicted values. Maximum FPA of 9.88 IU/mL and 8.38 IU/mL were obtained for *T. reesei* MCG 80 and QMY-1 respectively under the conditions of 4% NaOH pretreatment and initial culture pH 6, under solid-state fermentation conditions. Combination of NaOH pretreatment with steam explosion did not enhance the activity of the cellulase-systems of the two mutants. The production of inhibitory compounds with their consequent detrimental effects on the growth of the fermenting organisms was a possible cause. The cellulase-systems produced were capable of hydrolyzing over 80% delignified wheat straw.

4.2 INTRODUCTION

Cellulase production through fermentation technology and strain developments has led to some commercial application of cellulases in the food and fruit industries (Pathak and Ghose, 1973; Mandels, 1975) where small quantities are required. There is, however, the need to produce large quantities of cellulase-systems with high FPA and ßGA if large scale cellulose hydrolysis is to be economically viable for ethanol production. This need is amplified in the hydrolysis of lignocelluloses because limitation of either FPA or ßGA

causes end product inhibition which generally results in low sugar and ethanol yields. The importance of ß-glucosidase for instance can not be overemphasized because it is needed for both the final conversion of cellobiose to glucose and to relieve the end-product inhibition by this disaccharide towards endoglucanase.

More still, some cellulase-system activity is lost due to their adsorption onto lignin during hydrolysis of pretreated lignocelluloses and furthermore, enzyme recycling processes for the available processes for ethanol production either by separate hydrolysis and fermentation or by simultaneous hydrolysis and fermentation are not to date well developed.

Attempts have been made to overcome ß-glucosidase limitation through several approaches. One is the enrichment of the *T. reesei* cellulase with a β-glucosidase from analogous microorganism e.g., from *Aspergillus niger* (Bisset and Sternberg, 1978; Vernardo *et al.*, 1980). Another is to develop a mixed culture, the second being an effective β-glucosidase producer, e.g., *T. reesei* and *A. phoenicis* (Duff *et al.*, 1987, Awafo *et al.*; 1996) and also *T. reesei* and *A. wentii* (Ghose *et al.*, 1985). The third option is to utilize both culture supernatant and mycelium since β-glucosidase is membrane bound. This can present operational problems in the liquid fermentation cultures. In solid-state fermentation systems, the use of unextracted crude cellulase for hydrolysis is feasible and has several advantages (Awafo *et al.*; 1996). The last and very obvious approach is to find cellulolytic microorganisms that produce an effective cellulase that is rich in β-glucosidase. The literature seems to suggest cellulolytic species, like *Penicillium funiculosum* (Bastawade *et al.*, 1977), *Penicillium pinophilum* (Brown *et al.*, 1987), and

Sporotrichum pulverulentum (Durand and Tiraby, 1980; Eriksson and Johnsrud, 1983) are capable of producing cellulase-systems rich in ß-glucosidase though their levels of FPA are considerably lower than those obtained from *T. reesei* species.

Our approach is to develop a model to produce cellulase-systems from *T. reesei* mutants using wheat straw subjected to mild sodium hydroxide and in combination with steam explosion pretreatments to produce an effective cellulase-system that is also rich in β-glucosidase. Sodium hydroxide and steam explosion are two known pretreatments that have been reported in the literature. However, in almost all the reports, these pretreatments were used for the sole purpose of rendering the lignocelluloses suitable for enzymatic hydrolysis into hexose and pentose sugars. Not surprising, therefore, that these pretreatments were harsh either as a function of reaction temperature, holding time or pressure for steam explosion (Moniruzzaman, 1996; Beltrame *et al.*; 1992; Puri and Mamers, 1983); and high sodium hydroxide concentration /g substrate (Gray *et al.*; 1978; Gharpuray *et al.*; 1983; Choudhury *et al.*; 1984).

4.3 MATERIALS AND METHODS

4.3.1 Substrate Pretreatments

Ground wheat straw (20 mesh) was pretreated with sodium hydroxide and steam explosion and the fermentation substrate was prepared as outlined in the experimental design (Table 4-1)

4.3.2 Experimental Design

An optimization model of second order orthogonal composite design (Schmidt and Launsby, 1992; Box et la, 1978; Cochran and Cox, 1968) was used to study four factors for cellulase-system production with sodium hydroxide and its combination with steam explosion as pretreatments. In SSF, when sodium hydroxide is used as a pretreatment of wheat straw, three other factors, namely, steam sterilization, pH and urea are important in formulating the substrate for the growth of the microorganism and hence cellulase-system synthesis. Optimizing the level of sodium hydroxide for cellulase-system production is important since much of the literature contains information as it relates mostly to using sodium hydroxide to render the substrate susceptible to hydrolysis with external cellulase sources rather than fermentation for cellulase production (Choudhury *et al.*; 1984). When sodium hydroxide is used as a pretreatment of lignocelluloses, steam sterilization is an additional pretreatment to serve two functions; to enhance the alkali effects of fiber saturation and swelling capacity and to destroy microorganisms in the substrate to prevent them from contaminating the fermenting organism. *T. reesei* is known to require an optimum pH of about 6 for growth, however, in SSF, it is difficult to maintain this optimum temperature without pH controls for the sustained long duration of the fermentation. This factor is even more complicated when urea, a readily utilizable source of nitrogen, is present in the medium. The latter has the structural tendency to be much preferred as nitrogen source at the beginning of the fermentation and thus has the ability to raise up the pH of the fermentation system.

In the central composite orthogonal design with four factors (k=3), there were 8 kernel points (2^k), 6 star points α (at α = 1.682) and 6 replicates at the center point, all in duplicate to give a total of 40 sets per experiment as shown in Table 4-1. Two responses are studied in each experiment, namely, filter paper activity (FPA) and ß-glucosidase activity (ßGA).

In the study of sodium hydroxide pretreatment only, the design in Table 4-1 was used to optimize the NaOH concentration, steam sterilization time, and initial pH conditions for the production of the cellulase-systems of *T. reesei* QMY-1 and *T. reesei* MCG 80.

In the study of the combination pretreatment of sodium hydroxide with steam explosion, Table 4-1 was again employed to determine similar response parameters by optimizing NaOH concentration, temperature of steam explosion and holding time. Regression analysis was performed on the results obtained and used to fit a second-order polynomial equation to obtain an understanding of any possible interactions between or amongst the operating variables. A second-order equation of the form given below was fitted.

$$\text{FPA or ßGA} = ß_0 + ß_1x_1 + ß_2x_2 + ß_3x_3 + ß_{11}x_1^2 + ß_{22}x^2 + ß_{33}x^2 + ß_{12}x_1x_2 + ß_{13}x_1x_3 + ß_{23}x_2x_3 + ß_{123}x_1x_2x_3 \quad (1)$$

where

FPA or ßGA = predicted response

$ß_0$ = offset term;

$ß_1$, $ß_2$ and $ß_3$ = linear effect terms;

$ß_{11}$, $ß_{22}$ and $ß_{33}$ = squared effects; and

$ß_{12}$, $ß_{13}$, $ß_{23}$ and $ß_{123}$ = interaction effects.

The multiple coefficients of determination, R^2, obtained from the Analysis of variance (ANOVA) was used to explain the polynomial models. The computer software used for the study was Microsoft Excel version 7.0

Table 4-1: Orthogonal design matrix defining operating variables as they apply to pretreatments of wheat straw with NaOH and its combination with steam explosion.

Run no.	Operating variables		
	x_1	x_2	x_3
1	-1	-1	-1
2	-1	1	-1
3	-1	-1	1
4	-1	1	1
5	1	-1	-1
6	1	1	-1
7	1	-1	1
8	1	1	1
9	-1.682	0	0
10	1.682	0	0
11	0	-1.682	0
12	0	1.682	0
13	0	0	-1.682
14	0	0	1.682
15-20	0	0	0

The coded values,1, -1, 0, -1.682 and 1.682 are normalized values (dimensionless) (equation 2)

Table 4-2 shows the actual values of the treatment levels at the different normalized values for NaOH pretreatment alone. The normalized values are obtained from the following equation:

$$x_i = [X_i - (H_i + L_i/2)]/[(H_i - L_i/2)] \quad (2)$$

where, x_i = Normalized value (dimension less) of factor i , X_i = actual value of the independent variable i , H_i = upper limit of factor i, L_i = Lower limit of factor i, $(H_i + L_i/2)$ = actual value of the independent variable i at the center point, and $(H_i - L_i/2)$ = step change.

The independent variables were coded as

x_1=[NaOH (%) - 4]/2

x_2 = [t(mins) - 45]/15

x_3 = [pH - 6]/1

Table 4-2 Operating variables and their levels for sodium hydroxide pretreatment

Actual values	Coded levels				
	-α	Low	Center point	High	+α
	(-1.682)	(-1)	(0)	(+1)	(1.682)
NaOH (%w/w)	0	2	4	6	8
Sterilization time (min)	15	30	45	60	75
Initial pH	4	5	6	7	8

Table 4-3 shows the actual values of the treatment levels at the different normalized values for NaOH pretreatment in combination with steam explosion.

Table 4-3 Operating variables and their levels for sodium hydroxide pretreatment in combination with steam explosion at 20 MPa.

Actual values	Coded levels				
	-α (-1.682)	Low (-1)	Center point (0)	High (+1)	+α (+1.682)
NaOH (%w/w)	0	2	4	6	8
Temperature (T) (°C)	160	165	170	175	180
Holding time (t) (min)	3	5	6.5	8	10

The independent variables were coded as

x_1=[NaOH (%) - 4]/2

x_2 = [T(°C) - 170]/5

x_3 = [t(min) - 6.5]/1.5

4.3.3 Microorganisms

Trichoderma reesei QMY-1 (NRRL 18760) was obtained from Dr. D. S. Chahal (Institut Armand-Frappier, Laval, Québec, Canada) while *Trichoderma reesei* MCG 80 (NRRL 12368) was obtained from J.L. Swezey (ARS Patent Culture Collection, USDA, Peoria,

IL). Both organisms were maintained on modified Mandels medium agar slants with delignified wheat straw. The organisms were subcultured every two months and stored at 4 ± 1°C.

4.3.4 Cultivation Medium and Fermentation Conditions

Standard Mandels medium (Mandels and Weber, 1969) was used to prepare the pre-inoculum and inoculum of each test organism. Mycelial inoculum (36 h, in the exponential phase of growth) was used at the rate of 5 mL/4 g substrate (approximately 6.0×10^{-3} g mycelium/g substrate, dry weight basis). Mandels medium was the base medium for the fermentation experiments but was modified accordingly to obtain the conditions as outlined in Table 4-1 of the experimental design.

Modified Mandels medium (without urea) was added to each Erlenmeyer flask containing 4 g wheat straw (dry wt.) of each experimental set up in Table 4-1 and sterilized at 121°C for 30 min. The flasks were aseptically inoculated and placed in humidified incubators (Norco 4100) at 30°C for 3 weeks under solid-state fermentation conditions.

4.3.5 Enzyme Extraction and Analysis

The fermented substrate was extracted in a citrate buffer (pH, 4.8) with 0.1% Tween 80 first placed in a rotary shaker at 200 rpm for 30 min and then centrifuged at 11 000 g for 30 min. The supernatant was analyzed for FPA and ßGA (Mandels *et al.*, 1976, Ghose, 1987).

4.3.6 Hydrolytic Potential of Best Cellulase-System

The model used for the production of the cellulase-systems of *T. reesei* was validated by testing the hydrolytic potentials of the best cellulase-systems of the two mutants on the hydrolysis of delignified wheat straw, solka floka (α-cellulose), steam exploded wheat straw and untreated wheat straw. Delignified wheat straw was obtained by the method of Toyama and Ogawa (1972) while steam exploded wheat straw was the fractionated solid portion obtained from steam explosion under the following conditions: 4% NaOH, Steam explosion at 180°C for 6.5 min. Solka floka is a microcrystalline cellulose, obtained from the Sigma Chemical company.

Extracted cellulase-systems at the rate of 20 IU FPA/g substrate were added to 5% substrate in citrate buffer (0.1 M, pH 4.8) and incubated at 45°C for 60 h. The resulting sugars were analyzed with a Gold HPLC system with an attached Aminex HPX-87P column heated to 80°C and an Altex 156 Refractive Index Detector.

4.4 RESULTS AND DISCUSSION

4.4.1 Production of Cellulase-Systems of *T. reesei* QMY-1 and MCG 80 from Wheat Straw Pretreated with Sodium Hydroxide.

An effective cellulase-system should have enough activity to hydrolyze cellulose into glucose. This is achieved when the total activity of the endo- and exo-glucanases and ß-glucosidase components of the cellulase-system is such that the cellobiose units generated

by the endo- and exo-glucanases are completely converted into glucose by the ß-glucosidase component. The ratio of the activity of the latter to the combined activity of all the three enzyme components (FPA), is therefore, critical in attaining complete cellulose hydrolysis. The ability of the cellulase-system to hydrolyze microcrystalline cellulose such as Whatman filter paper is used as a measure of the cellulase-system activity (Ghose, 1978).

4.4.2 Filter paper activity (FPA) of the Cellulase-Systems of *T. reesei* QMY-1 and *T. reesei* MCG 80 obtained from Wheat Straw Pretreated with Sodium Hydroxide

Tables 4-4 and 4-8 show the respective experimental results of the FPA of *T. reesei* QMY-1 and MCG 80 cultivated on wheat straw pretreated with sodium hydroxide. Both mutants showed maximum FPA at initial pH 6 and 4% NaOH pretreated wheat straw. Maximum FPA of 8.38 IU/mL and 9.88 IU/mL were obtained from *T. reesei* QMY-1 and MCG 80 respectively. Sterilization of the substrate either for the minimum time of 15 min or up to 75 min did not appear to affect the optimum FPA produced by these two mutants. However, a low pH of 4 and high pH of 8 were both detrimental to the production of FPA. These conditions were observed as virtual absence of growth on these cultures under the solid-state fermentation conditions studied.

By applying multiple regression analysis on the experimental data, the second-order polynomial equation obtained for the FPA from *T. reesei* QMY-1 was

$$FPA = 4.99 + 0.53x_1 + 0.76x_3 + 1.05x_2^2 - 1.25x_3^2 \quad (3)$$

and for the FPA obtained from *T. reesei* MCG 80,

$$FPA = 5.58 + 0.86x_3 + 1.35x_2^2 - 1.60x_3^2 \quad (4)$$

Tables 4-4 and 4-8 also show the predicted FPA from equations (3) and (4). The multiple coefficients of correlation, R, for the FPA of *T. reesei* QMY-1 and MCG 80 are respectively, 0.979 and 0.957 suggesting a close agreement between experimental and predicted FPA values. The multiple coefficients of determination, R^2, show that 95.8% of the sample variations for FPA from *T reesei* QMY-1 and 91.6% for FPA from *T. reesei* MCG 80 are attributable to the operating variables, namely, sodium hydroxide concentration, sterilization time and initial pH.

The analysis of variance (ANOVA) corresponding to the experimental data of Tables 4-4 and 4-8 are presented in Tables 4-5 and 4-9. The *F* values for the FPA of *T. reesei* QMY-1 and MCG 80 were 20.77 and 12.52, respectively. These values were greater than *F* (10, 9) within a rejection region having an α-level of $P < 5.01 \times 10^{-5}$ and 3.97×10^{-4}, respectively.

4.4.3 ß-Glucosidase Activity (ßGA) of the Cellulase-Systems of *T. reesei* QMY-1 and MCG 80 obtained from Wheat Straw Pretreated with Sodium Hydroxide.

The ßGA of *T. reesei* QMY-1 and MCG 80 appeared to be maximum, 7.3 IU/mL and 7.97 IU/mL respectively, under the conditions when wheat straw was pretreated with 4% NaOH, sterilized for 45 min and an initial pH of 6 (Tables 4-6 and 4-10). These

conditions are quite identical to those that were needed to obtain maximum FPA. Extremes of pH (4) and NaOH concentration (8%) were again not suitable for the synthesis of ß-glucosidase.

Second-order polynomial equations (5) and (6), were obtained by multiple regression analysis of the experimental data in Tables 4-6 and 4-10. The ßGA from *T. reesei* QMY-1 was

$$ßGA = 6.93 - 1.71x_1^2 - 0.66x_2^2 - 2.31x_3^2 - 1.21x_2x_3 \quad (5)$$

and that for the ßGA obtained from *T. reesei* MCG 80 was

$$ßGA = 7.43 + 0.87x_3 - 1.7x_1^2 - 2.39x_3^2 + 1.06x_1x_3 \quad (6)$$

The predicted ßGA from equations (5) and (6) are also shown in Tables 4-6 and 4-10. The multiple coefficients of correlation, R, for the ßGA of *T. reesei* QMY-1 and MCG 80 are respectively, 0.972 and 0.957 which indicates a close agreement between the experimental ßGA and the predicted ßGA values. The multiple coefficients of determination, R^2, show that 94.6% and 91.6% of the sample variations for the respective ßGA of *T. reesei*

QMY-1 and MCG 80 were attributable to the independent factors *viz.*, sodium hydroxide concentration, sterilization time and initial pH. The corresponding ANOVA are shown in Tables 4-7 and 4-11. The *F* values of 15.87 and 9.83 for the ßGA of *T. reesei* QMY-1 and MCG 80 respectively, were greater than $F(10, 9)$ within a rejection region with α-level of $P < 1.5 \times 10^{-4}$ and 1.03×10^{-3}, respectively.

4.4.4 Filter Paper Activity of the Cellulase-System of *T. reesei* QMY-1 and MCG 80 obtained from Wheat Straw Pretreated with a Combination of Sodium Hydroxide and Steam Explosion.

Mild sodium hydroxide pretreatment alone has the effect of increasing the swelling capacity and fiber saturation point of the substrate and thus increases the accessibility of the substrate to the fermenting organism. Steam explosion on the other hand, increases accessibility of the substrate by structurally modifying it by wetting, increasing its pore size and bulk surface area. The combined effects of these pretreatments on the production of cellulase-systems of *T. reesei* QMY-1 and MCG 80 are shown in Tables 4-12 to 4-18. Tables 4-12 and 4-16 show the respective results of the FPA of *T. reesei* QMY-1 and MCG 80 which both indicate that maximum FPA (7.8 and 7.54 IU/mL) were obtained when the wheat straw was pretreated with 4% NaOH and subjected to steam explosion at 170°C for 6.5 minutes. The FPA of both mutants were not enhanced when the operating parameters were kept either at their low or high levels.

Multiple regression analysis of the experimental results produced the following second-order polynomial equations for the FPA of both mutants. The equation for the FPA from *T. reesei* QMY-1 was

$$FPA = 7.54 + 0.67x_2 - 1.12x_1^2 - 1.79x_2^2 - 0.39x_3^2 - 0.67x_1x_2 - 1.63x_1x_3 + 1.62x_1x_2x_3 \quad (7)$$

and that for the FPA obtained from *T. reesei* MCG 80 was

$$FPA = 6.62 + 0.52x_2 - 0.71x_1^2 - 1.07x_2^2 - 1.17x_1x_3 + 1.08x_1x_2x_3 \quad (8)$$

The multiple coefficients of correlation, R, for the FPA of *T. reesei* QMY-1 and MCG 80 are respectively, 0.986 and 0.981 which implies a close agreement between experimental and predicted FPA values. The multiple coefficients of determination, R^2, show that 97.2% and 96.2% of the sample variations for the respective ßGA of *T. reesei* QMY-1 and MCG 80 were attributable to the independent factors *viz.*, sodium hydroxide concentration, temperature of steam explosion and holding time. The corresponding ANOVA are shown in Tables 4-13 and 4-17. The *F* values of 15.87 and 9.83 for the ßGA of *T. reesei* QMY-1 and MCG 80 respectively, were greater than $F(10, 9)$ within a rejection region with α-level of $P < 3.6\times 10^{-45}$ and 1.32×10^{-4}, respectively.

4.4.5 ß-Glucosidase Activity (ßGA) of the Cellulase-Systems of *T. reesei* QMY-1 and MCG 80 obtained from Wheat Straw Pretreated with Sodium Hydroxide in Combination with Steam Explosion

Maximum ßGA of 3.02 IU/mL and 3.94 IU/mL were obtained from *T. reesei* QMY-1 and MCG 80 respectively. Both mutants produced maximum ßGA when the wheat straw was subjected to steam explosion at 170°C for 6.5 min with 4% NaOH pretreatment (Tables 4-14 and

4-18). These conditions are similar to those that were needed to obtain maximum FPA. Extremes of NaOH concentration (0% or 8%), explosion temperature (160°C or 180°C), and holding time (3 min or 10 min) were not suitable for the synthesis of ß-glucosidase.

Second-order polynomial equations (9) and (10), were obtained by multiple regression analysis of the experimental data in Tables 4-6 and 4-10. The ßGA from *T. reesei* QMY-1 was

$$ßGA = 2.38 + 0.35x_1 + 0.49x_2 - 0.33x_1^2 - 0.24x_2^2 - 0.89x_3^2 + 0.28x_1x_2 - 0.29x_1x_3 - 0.32x_2x_3 - 0.34x_1x_2x_3 \quad (9)$$

and that for the ßGA obtained from *T. reesei* MCG 80 was

$$ßGA = 3.73 + 0.30x_1 + 0.32x_2 - 0.54x_1^2 - 0.48x_2^2 - 1.24x_3^2 - 0.31x_1x_3 - 0.25x_2x_3 - 0.28x_1x_2x_3 \quad (10)$$

The multiple coefficients of correlation, R, for the ßGA of *T. reesei* QMY-1 and MCG 80 are respectively, 0.977 and 0.99 which indicates very close agreement between the experimental ßGA and the predicted ßGA values (Tables 4-14 and 4-18). The multiple coefficients of determination, R^2, show that 95.5% and 98% of the sample variations for the respective ßGA of *T. reesei* QMY-1 and MCG 80 were attributable to the independent factors *viz.*, sodium hydroxide concentration, temperature of steam explosion and holding time. The corresponding ANOVA are shown in Tables 4-15 and 4-19. The *F* values of 17.19 and 39.32 for the ßGA of *T. reesei* QMY-1 and MCG 80 respectively, were greater than F(10, 9) within a rejection region with α-level of $P < 2.4\times 10^{-4}$ and 1.04×10^{-5}, respectively.

4.4.6 Comparison of the Activities of the Cellulase-Systems of *T. reesei* QMY-1 and MCG 80 obtained from both Pretreatments.

Covariance analysis of the FPA and ßGA of the cellulase-systems of both of *T. reesei* QMY-1 and MCG 80 obtained from the wheat straw pretreated with NaOH showed that there were no significant differences at $P \leq 0.05$ in the levels of these activities for both mutants. This was similarly the case with wheat straw pretreated with both NaOH and steam explosion. However, comparison of the FPA and ßGA of the two mutants obtained from the two pretreatments of NaOH alone and in combination with steam explosion showed significant differences in the levels of FPA and ßGA at $P \leq 0.05$. The FPA and ßGA obtained from the two mutants at all levels of treatments were significantly higher ($P \leq 0.05$) with wheat straw pretreated with NaOH alone than with the combination of NaOH and steam explosion. The plausible explanation of the observed phenomenon is that the combination pretreatment most likely produced more inhibitory substances than with sodium hydroxide alone. Steam explosion is known to produce inhibitory substance such as phenol-like compounds, furfural, and hydroxymethyl furfural (Neese *et al.*, 1977; Campbell *et al.*, 1973).

Table 4-20 shows summary of some optimum cellulase-system activities from the two different pretreated wheat straw substrate. Optimum cellulase-system activities of about 9.88 FPA in IU/mL (247 IU/g) and 6.38 IU/mL (159 IU/g) ßGA were obtained from wheat straw pretreated with 4% NaOH, sterilized for 45 min at pH 6

4.4.7 Validation of Optimization Model: Hydrolytic Potential of Best Cellulase-Systems of *T. reesei* QMY-1 and MCG 80.

The hydrolytic potentials of the best cellulase-systems of *T. reesei* QMY-1 (FPA, 8.38 IU/mL; ßGA, 5.5 IU/mL) and *T. reesei* MCG 80 (FPA, 9.88 IU/mL; ßGA, 6.38 IU/mL) were subjected to hydrolysis of 5% substrate and the results are shown in Table 4-21. The results demonstrate that out of the four substrates tested, delignified wheat straw was most preferred while untreated wheat straw and α-cellulose were the least preferred substrates for hydrolysis. This supports the fact that pretreatment of cellulose is a pre-requisite for effective hydrolysis. The hydrolytic potentials of the cellulase-system from *T. reesei* MCG 80 on all the substrates were slightly higher than those from *T. reesei* QMY-1. This has a direct reflection on the fact that the cellulase-system of *T. reesei* MCG 80 had higher FPA and ßGA than its counterpart, *T. reesei* QMY-1. More still, unextracted cellulase-systems from both mutants out performed the extracted cellulase-systems to give more reducing sugars suggesting the former has better potential commercial applications in cellulose hydrolysis since it also has the added advantage of reducing enzyme cost because the enzyme extraction process could be eliminated altogether.

4.5 CONCLUSION

The optimization model used for the production of the cellulase-systems of *T. reesei* QMY-1 and MCG 80 appears to be a viable tool for ascertaining optimum conditions for

the pretreatment of wheat straw for maximum cellulase-system activity under the parameters studied. Extremes of pH (4 and 8) and high sodium hydroxide concentration (8%) appear to be detrimental in cellulase-system production. Crude cellulase-systems of both mutants were also efficient at hydrolyzing over 80% of delignified wheat straw.

Table 4-4: Filter paper activity of the cellulase-system of *T. reesei* QMY-1 under SSF conditions with wheat straw pretreated with sodium hydroxide

	Filter Paper Activity (FPA)(IU/mL)			
	Experimental			
Run no.	I	II	Mean (FPA)	Predicted (FPA)
1	2.74	2.29	2.52	2.96
2	2.91	3.35	3.13	3.62
3	5.46	4.68	5.07	5.04
4	5.10	4.68	4.89	4.91
5	3.77	4.22	4.00	4.46
6	3.78	4.35	4.07	4.57
7	6.02	7.00	6.51	6.51
8	5.65	4.74	5.20	5.24
9	4.16	3.96	4.06	3.74
10	6.62	5.17	5.90	5.53
11	8.74	8.02	8.38	8.10
12	8.04	8.41	8.22	7.83
13	1.21	0.90	1.06	0.16
14	2.10	2.91	2.51	2.72
15	5.41	4.51	4.96	4.99
16	4.53	4.78	4.65	4.99
17	5.03	5.23	5.13	4.99
18	4.33	5.34	4.83	4.99
19	5.36	5.01	5.19	4.99
20	4.79	5.35	5.07	4.99

R, 0.979; R^2, 0.958

Table 4-5: ANOVA table for the FPA of *T. reesei* QMY-1 under SSF conditions with wheat straw pretreated with sodium hydroxide

Source	df	SS	MS	F	Significance F
Regression	10	55.877	5.588	20.771	5.01×10^{-5}
Residual	9	2.421	0.269		
Total	19	58.298			

df, degrees of freedom; SS, sum of squares; MS, mean square

Table 4-6: ß-Glucosidase activity of the cellulase-system of *T. reesei* QMY-1 under SSF conditions with wheat straw pretreated with sodium hydroxide

ß-Glucosidase activity (ßGA)(IU/mL)				
	Experimental			
Run no.	I	II	Mean (ßGA)	Predicted (ßGA)
1	0.60	0.69	0.64	1.11
2	1.67	1.85	1.76	2.87
3	2.58	2.05	2.31	2.04
4	0.98	0.80	0.89	1.26
5	0.60	0.64	0.62	0.89
6	1.09	1.35	1.22	2.13
7	5.98	6.50	6.24	6.77
8	0.80	0.62	0.71	0.88
9	2.96	1.18	2.07	1.38
10	3.18	2.87	3.02	2.81
11	5.740	5.300	5.520	5.83
12	6.07	4.93	5.50	4.28
13	0.79	0.99	0.89	0.44
14	0.890	0.706	0.798	1.22
15	6.97	7.15	7.06	6.93
16	6.05	6.23	6.14	6.93
17	5.98	7.02	6.50	6.93
18	7.15	7.45	7.30	6.93
19	7.00	7.48	7.24	6.93
20	7.52	6.83	7.18	6.93

R, 0.972; R^2, 0.946

Table 4-7: ANOVA table for the ßGA of *T. reesei* QMY-1 under SSF conditions with wheat straw pretreated with sodium hydroxide

Source	df	SS	MS	F	Significance F
Regression	10	140.666	14.0667	15.873	1.5×10^{-4}
Residual	9	7.976	0.886		
Total	19	148.642			

df, degrees of freedom; SS, sum of squares; MS, mean square

Table 4-8: Filter paper activity of the cellulase-system of *T. reesei* MCG 80 under SSF conditions with wheat straw pretreated with sodium hydroxide

	Filter Paper Activity (FPA)(IU/mL)			
	Experimental			
Run no.	I	II	Mean (FPA)	Predicted (FPA)
1	2.63	2.29	2.46	3.31
2	2.48	4.43	3.46	4.12
3	6.78	5.64	6.21	6.16
4	4.76	6.14	5.45	5.22
5	3.52	3.95	3.74	4.22
6	4.87	4.45	4.66	4.96
7	6.53	7.48	7.00	6.59
8	6.35	5.90	6.12	5.53
9	4.62	4.94	4.78	4.17
10	5.76	4.18	4.97	5.22
11	9.39	10.38	9.88	9.48
12	9.16	9.35	9.25	9.29
13	1.00	0.71	0.86	0.39
14	2.01	1.22	1.61	2.50
15	5.45	5.97	5.71	5.58
16	5.25	5.45	5.35	5.58
17	4.72	5.95	5.34	5.58
18	4.70	5.09	4.90	5.58
19	6.54	5.48	6.01	5.58
20	5.00	7.20	6.10	5.58

R, 0.966; R^2, 0.933

Table 4-9: ANOVA table for the FPA of *T. reesei* MCG 80 under SSF conditions with wheat straw pretreated with sodium hydroxide

Source	df	SS	MS	F	Significance F
Regression	10	84.284	8.428	12.521	3.97×10^{-4}
Residual	9	6.059	0.673		
Total	19	90.342			

df, degrees of freedom; SS, sum of squares; MS, mean square

Table 4-10: ß-Glucosidase activity of the cellulase-system of *T. reesei* MCG 80 under SSF conditions with wheat straw pretreated with sodium hydroxide

	ß-Glucosidase activity (ßGA)(IU/mL)			
	Experimental			
Run no.	I	II	Mean (ßGA)	Predicted (ßGA)
1	0.51	0.60	0.55	1.80
2	1.81	1.99	1.90	3.13
3	2.79	2.25	2.52	2.46
4	1.88	1.70	1.79	1.71
5	0.60	0.64	0.62	0.89
6	0.99	1.03	1.01	1.50
7	5.96	6.48	6.22	5.40
8	6.65	6.47	6.56	5.73
9	3.56	1.78	2.67	1.48
10	4.30	1.98	3.14	3.75
11	6.60	6.16	6.38	6.07
12	8.36	5.42	6.89	6.62
13	0.93	1.13	1.03	0.81
14	0.96	0.77	0.86	2.13
15	7.65	7.83	7.74	7.43
16	7.52	7.70	7.61	7.43
17	6.89	7.93	7.41	7.43
18	6.70	7.00	6.85	7.43
19	6.64	7.11	6.87	7.43
20	8.32	7.63	7.97	7.43

R, 0.957; R^2, 0.916

Table 4-11: ANOVA table for the ßGA of *T. reesei* MCG 80 under SSF conditions with wheat straw pretreated with sodium hydroxide.

Source	df	SS	MS	F	Significance F
Regression	10	140.875	14.087	9.826	1.03×10^{-3}
Residual	9	12.903	1.434		
Total	19	153.778			

df, degrees of freedom; SS, sum of squares; MS, mean square

Table 4-12: Filter paper activity of the cellulase-system of *T. reesei* QMY-1 under SSF conditions with steam pretreated wheat straw

	Filter Paper Activity (FPA)(IU/mL)			
	Experimental			
Run no.	I	II	Mean (FPA)	Predicted (FPA)
1	0.21	0.31	0.26	0.02
2	7.07	7.26	7.17	7.41
3	5.87	5.35	5.62	5.83
4	3.44	3.26	3.35	4.06
5	6.72	6.77	6.74	6.26
6	0.50	0.76	0.63	0.64
7	5.57	6.09	5.83	5.80
8	3.60	3.42	3.51	3.98
9	5.23	3.45	4.34	4.77
10	5.00	4.49	4.74	4.00
11	1.19	0.75	0.97	1.36
12	4.08	4.53	4.31	3.60
13	6.92	7.12	7.02	6.58
14	6.27	6.09	6.18	6.31
15	7.08	7.26	7.17	7.54
16	7.69	7.87	7.78	7.54
17	7.28	8.32	7.80	7.54
18	7.41	7.71	7.56	7.54
19	7.11	7.59	7.35	7.54

R, 0.986; R^2, 0.972

Table 4-13: ANOVA table for the FPA of *T. reesei* QMY-1 under SSF conditions with steam pretreated wheat straw

Source	df	SS	MS	F	Significance F
Regression	10	106.684	10.668	28.387	3.62×10^{-5}
Residual	8	3.007	0.376		
Total	18	109.691			

df, degrees of freedom; SS, sum of squares; MS, mean square

Table 4-14: ß-Glucosidase activity of the cellulase-system of *T. reesei* QMY-1 under SSF conditions with steam pretreated wheat straw

ß-Glucosidase activity (ßGA)(IU/mL)				
	Experimental			
Run no.	I	II	Mean (ßGA)	Predicted (ßGA)
1	0.10	0.20	0.15	0.21
2	0.16	0.14	0.15	0.22
3	0.78	0.25	0.51	0.57
4	2.11	1.93	1.02	2.11
5	0.28	0.32	0.30	0.50
6	0.50	0.55	0.52	0.74
7	0.69	0.81	0.75	0.97
8	0.81	0.64	0.73	0.96
9	1.14	0.96	1.05	0.86
10	2.41	2.09	2.25	2.03
11	1.27	0.83	1.05	0.86
12	3.22	2.28	2.75	2.52
13	0.09	0.07	0.08	0.05
14	0.04	0.02	0.03	0.35
15	2.71	2.89	2.80	2.38
16	2.16	2.34	2.25	2.38
17	1.83	2.04	2.25	2.38
18	2.30	2.60	2.45	2.38
19	2.13	2.60	2.36	2.38

R, 0.977; R^2, 0.955

Table 4-15: ANOVA table for the ßGA of *T. reesei* QMY-1 under SSF conditions with steam pretreated wheat straw

Source	df	SS	MS	F	Significance F
Regression	10	19.782	1.978	17.194	2.37×10^{-4}
Residual	8	0.920	0.115		
Total	18	20.702			

df, degrees of freedom; SS, sum of squares; MS, mean square

Table 4-16: Filter paper activity of the cellulase-system of *T. reesei* MCG 80 under SSF conditions with steam pretreated wheat straw

	Filter Paper Activity (FPA)(IU/mL)			
	Experimental			
Run no.	I	II	Mean (FPA)	Predicted (FPA)
1	2.41	2.499	2.45	2.15
2	6.79	6.97	6.88	7.00
3	6.56	6.02	6.29	6.23
4	5.04	4.86	4.95	5.30
5	7.02	7.07	7.04	6.53
6	2.31	2.57	2.44	2.35
7	4.97	6.49	6.23	5.95
8	4.62	4.44	4.53	4.67
9	5.01	3.63	4.32	4.93
10	5.03	4.32	4.67	4.29
11	2.54	2.10	2.32	2.72
12	5.00	4.24	4.62	4.45
13	7.44	7.64	7.54	7.39
14	6.61	6.43	6.52	6.89
15	5.97	6.15	6.06	6.62
16	6.67	6.85	6.76	6.62
17	6.33	7.37	6.85	6.62
18	6.45	6.75	6.60	6.62
19	6.62	7.09	6.85	6.62

R, 0.981; R^2, 0.962

Table 4-17: ANOVA table for the FPA of *T. reesei* MCG 80 under SSF conditions with steam pretreated wheat straw

Source	df	SS	MS	F	Significance F
1.03×10^{-3}	10	48.258	4.826	20.117	1.32×10^{-4}
Residual	8	1.919	0.240		
Total	18	50.177			

df, degrees of freedom; SS, sum of squares; MS, mean square

Table 4-18: ß-Glucosidase activity of the cellulase-system of *T. reesei* MCG 80 under SSF conditions with steam pretreated wheat straw

	ß-Glucosidase activity (ßGA)(IU/mL)			
	Experimental			
Run no.	I	II	Mean (ßGA)	Predicted (ßGA)
1	0.79	0.88	0.84	1.03
2	0.86	1.04	0.95	0.94
3	0.48	0.34	0.41	0.85
4	3.38	3.20	3.29	3.40
5	1.21	1.26	1.23	1.39
6	1.18	1.44	1.31	1.20
7	1.25	1.77	1.51	1.37
8	1.56	1.39	1.48	1.52
9	1.01	1.24	1.12	1.69
10	1.66	1.35	1.50	2.71
11	2.02	1.58	1.80	1.81
12	1.90	1.09	1.50	2.90
13	0.59	0.79	0.69	0.38
14	0.06	0.04	0.05	0.07
15	3.56	3.74	3.65	3.73
16	3.85	4.03	3.94	3.73
17	3.38	4.42	3.90	3.73
18	4.01	4.31	4.16	3.73
19	3.79	4.27	4.03	3.73

R, 0.99; R^2, 0.98

Table 4-19: ANOVA table for the ßGA of *T. reesei* MCG 80 under SSF conditions with steam pretreated wheat straw

Source	df	SS	MS	F	Significance F
Regression	10	29.525	2.952	39.322	1.04×10^{-5}
Residual	8	0.601	0.075		
Total	18	30.126			

df, degrees of freedom; SS, sum of squares; MS, mean square

Table 4-20 : Comparison of cellulase-system activities from pretreated wheat straw obtained from two different optimization models

Pretreatment of wheat straw	Fermenting microorganism	Cellulase-system activity			
		FPA		ßGA	
		(IU/mL)	(IU/g)	(IU/mL)	(IU/g)
4% NaOH, 45 min sterilization, pH, 6	*T. reesei* QMY-1	8.38	209	5.52	138
4% NaOH, 45 min sterilization, pH, 6	*T. reesei* MCG 80	9.88	247	6.38	159
4% NaOH, steam at 170°C, holding time of 6.5 min	*T. reesei* QMY-1	7.80	195	2.25	56
4% NaOH, steam at 170°C, holding time of 6.5 min	*T. reesei* MCG 80	6.85	171	3.90	97

Table 4-21: Hydrolytic potentials of different cellulase-systems on different substrates at 5% concentration.

Cellulase-system	Delignified Wheat straw (WS)		Steam Pretreated WS		α- Cellulose		Untreated WS	
	Total sugars (g/L)	Hydrolysis (%)	Total sugars (g/L)	Hydrolysis (%)	Total sugars (g/L)	Hydrolysis (%)	Total sugars (g/L)	Hydrolysis (%)
T. reesei QMY-1, Extracted	30.8	56	22.1	40.2	13.6	27	2.8	5
T. reesei QMY-1, Unextracted	45.0	82	26.9	49	14.9	30	3.4	6
T. reesei MCG 80, Extracted	48.5	88	33.0	60	21.2	42	3.5	6
T. reesei MCG 80, Unextracted	53.9	98	38.9	71	22.6	45	3.9	7

CHAPTER 5

EVALUATION OF A NOVEL PAN-BIOREACTOR FOR CELLULASE-SYSTEM BIOSYNTHESIS UNDER SOLID-STATE FERMENTATION CONDITIONS

5.0 CONNECTING STATEMENT

Once conditions were optimized for cellulase-system production (chapter 4) attempts were made to determine the extent to which production could be scaled-up. To this end a pan-bioreactor designed by Dr. D.S. Chahal and Dr. P.S. Chahal was evaluated for its capacity to support different substrate concentrations for the production of cellulase-systems. The quality of the cellulase-system was assessed.

Note : This chapter constitutes the text of a paper submitted for publication as follows :

Awafo, V.A., Chahal, D.S. and Simpson B.K. 1997. Evaluation of a novel pan-bioreactor for cellulase-system biosynthesis under solid-state fermentation conditions. Biotechnol. Bioeng.

Contribution of Co-authors : Chahal, D.S. and Simpson, B.K. (Thesis co-supervisors)

Part of this chapter has also been presented at the 18th Symp. On Biotechnol. For Fuels and Chemicals

5.1 ABSTRACT

The biosynthesis of cellulase-systems for cellulose hydrolysis is a major cost component in the production of fuel ethanol from lignocelluloses. A prototype pan-bioreactor for solid-state fermentation was capable of producing high cellulase activities on wheat straw subjected to two pretreatments of mild sodium hydroxide and steam explosion. The pan-bioreactor could support high substrate concentrations up to 50g /pan, though the highest cellulase activities were achieved with 10 and 20 g substrate/pan-bioreactor.

Well defined process parameters of initial enzyme concentration, initial substrate concentration and batch hydrolysis time were established in evaluating the suitability of steam pretreated and washed substrate for the production of sugars. The cellulase-system of two test organisms assessed showed that enzyme loading of at least 30 IU/g obtained from *T. reesei* MCG 80 were required for over 80% hydrolysis of 2-5% steam pretreated wheat straw. Increasing the substrate concentration beyond 5% resulted in lower hydrolysis even when the enzyme loading was increased.

5.2 INTRODUCTION

Increased awareness of the limitations of liquid-state fermentation to produce a complete cellulase-system for cellulose hydrolysis has spurred interest in the development of bioreactors for cellulase-system production in solid-state fermentation (SSF) systems. A

cellulase-system contains at least three main enzyme components namely; endo-, exo-glucanases and ß-glucosidase and a complete cellulase-system is defined as one with the ratio of ß-glucosidase activity (ßGA) to filter paper activity (FPA) close to one. FPA is the expressed synergistic action of all the three cellulase-system components during cellulose hydrolysis. The proportion of ßGA- to- FPA is important in alleviating cellobiose accumulation in order to achieve complete cellulose hydrolysis (Stockton *et al.*, 1991; Breuil *et al.*, 1992). Bioreactors that have been used for cellulase-system production include rotary trays and drums, stationary trays and moving belts (Toyama, 1976; Silman, 1980). The growth of cellulase-system producing organisms like *Trichoderma reesei* is severely hindered as mixing occurs in most of these bioreactors, thus limiting their ability to produce sufficient quantities of cellulases in the right amounts for complete cellulose hydrolysis. It is postulated that as more economical systems are sought for cellulase-system production, and as SSF gains more popularity for its ability to produce cellulase-systems, then better bioreactor designs will be needed. It was in this vein that a pan-bioreactor was designed to overcome the mixing and aeration problems encountered in most other solid-state fermentation systems and to enhance cellulase-system production. The prototype pan-bioreactor (16 x 16 x 8 cm^3) (Fig. 5-1) was designed by. D.S. Chahal and P. S. Chahal and constructed with stainless steel with support bars in the middle of the pan to house a replaceable screen (60 mesh) or glass plate. Wheat straw was chosen as the substrate for the enzyme production and cellulose hydrolysis because it represents a renewable and low cost energy resource that is available in significant amounts in many regions of the world (Ishaque and Chahal, 1991). Wheat straw also represents a good example of lignocellulosic substrates in terms

of basic or principal composition namely, cellulose (39%), hemicelluloses (36%), and lignin (10%).

In this study, the pan-bioreactor was evaluated for its ability to support the growth of *T. reesei* on pretreated wheat straw for cellulase-system production and to provide information on optimum substrate loading in an incremental step towards the possibility of scaling up cellulase-system production under SSF conditions. The quality of the cellulase-system produced was further evaluated by optimizing the cellulase-system loading and substrate concentration needed to attain optimum cellulose hydrolysis. The latter study was predicated against the background that clear process parameters, namely, initial substrate concentration, initial enzyme concentration and batch time have to be defined for any substrate used for hydrolysis so as to minimize the production cost of sugar.

5.3 MATERIALS AND METHODS

5.3.1 Microorganisms

Trichoderma reesei mutants QMY-1 (NRRL 18760) and MCG 80 (NRRL 12368) were maintained on agar slants of modified Mandels medium (Mandels and Weber, 1969) with delignified wheat straw as carbon source. *T. reesei* QMY-1 was kindly provided by D. S. Chahal, Institut Armand-Frappier, Laval, Quebec, and *T. reesei* MCG 80 was supplied by J.L. Swezey, ARS Patent Culture Collection, USDA, Illinois, USA. Both cultures were maintained at 4 ± 1°C.

5.3.2 Fermentation Substrate

Two lignocellulosic materials were used as fermentation substrates; wheat straw pretreated with 4% NaOH and sterilized at 121°C for 1h and wheat straw subjected to steam explosion at 190°C for 4 min and washed. The first substrate contained both cellulose and hemicelluloses while the latter contained mainly cellulose.

5.3.3 Pan-bioreactor and Substrate Loading

The pan-bioreactor (Fig 5-1) was assessed for its ability to contain various concentrations of substrate on dry weight basis (dwb) from 10 - 50 g substrate / pan-bioreactor for the growth and production of cellulase-systems from *T. reesei* QMY-1 and MCG 80. Each pan-bioreactor contained the fermentation substrate either spread on the screen (60 mesh) support in the middle of the bioreactor or spread on the glass support. The mesh screen support permitted flow of air throughout the fermentation bed while air flow was restricted in the fermentation bed with glass support. Mandels medium components (without glucose) were calculated per gram substrate and added to each pan-bioreactor. Water was added to bring the final moisture content to 75% and then sterilized at 121°C for 30 min.

5.3.4 Cellulase-System Production and Extraction

T. reesei QMY-1 and MCG 80 were initially cultivated on standard Mandels medium for 72 h and transferred at the rate of 10% (v/v) to fresh Mandels medium with 2% glucose for 36 h. Each test organism was aseptically inoculated on the surface of the pan-bioreactors on the basis of 5mL/4 g substrate. The samples were kept in aerated stationary

humidified incubators (Norcoo 4100, New Brunswick Scientific, N.J.) for 21 days. The harvested enzymes were extracted by adding 0.1M citrate buffer, (pH 4.8) to 4 g fermented substrate (dwb) to a final volume of 100 mL water and centrifuged at 11 000 g. The supernatant was then analyzed for its filter paper activity (FPA) and ß-glucosidase activity (ßGA) according to IUPAC protocol (Ghose, 1987; Mandels *et al.*, 1976). The cellulase yields per gram were obtained by dividing the total international units of cellulase activity in the extraction volume by the quantity of substrate (dwb) taken from the pan-bioreactor for extraction.

5.3.5 Cellulose Hydrolysis

Based on the cellulase-system activity from either *T. reesei* QMY-1 or MCG 80 obtained from the fermenting substrates, the best cellulase-system activities from *T. reesei* QMY-1 and MCG 80 at 20 IU/g substrate were used for the hydrolysis of 5% steam pretreated wheat straw (SWS) over a period of time.

A 2^2 factorial model was then applied to optimize both enzyme concentration and SWS concentration for maximum sugar production after 96 h hydrolysis. All the hydrolysis was performed at 45°C in 0.05 M citrate buffer at pH 4.8. The individual reducing sugars were determined by HPLC (Beckman Gold system) with an Aminex HPX-87P column (Biorad) coupled to an Altex 156 Refractive Index detector. The flow rate was set at 0.6 mL/min in a deionized water mobile phase heated to 80 °C.

5.3.6 Microstructure Studies

Three samples; unpretreated wheat straw, NaOH pretreated wheat straw, and cellulase hydrolyzed wheat straw were subjected to scanning electron microscopy by desiccating the samples at 40°C in an air oven and storing the samples over P_2O_5. The samples were subjected to metallic shadow casting in a Polaron E-5000 system to cover the material with 350 nm thickness Au-Pb layer. The samples were then observed in an ISI-SS60 scanning electron microscope apparatus.

5.4 RESULTS AND DISCUSSIONS

5.4.1 Pan-bioreactor and Cellulase Production

Tables 5-1 to 5-4 show the production of cellulase-systems from *T. reesei* QMY-1 and MCG 80 cultivated on wheat straw pretreated with 4% sodium hydroxide and steam. Each Table shows that the activities of cellulase-systems obtained from pan-bioreactors with mesh screen were not significantly different from those obtained from pan-bioreactors having glass supports ($p \leq 0.05$) but the cellulase activities in both supports decreased with concentration of substrate especially after 20 g / pan. These two findings would suggest that once the fermentation bed is thin enough for aeration (~ 0.5 - 1.0 cm^3) then the type of support may not be very important. It should, however, be remarked that the fermentation time was limited to 21 days and that may not have been sufficient time for the microorganisms to utilize much of the substrate at higher concentrations for cellulase-system production. The FPA (IU/mL) and ßGA (IU/mL) obtained from 10 and 20 g NWS/pan-bioreactor were comparable to those obtained with similar substrate at 4

g/ Erlenmeyer flask in chapter 4 suggesting that SSF could be scaled-up in the novel pan-bioreactor. The obvious advantage with the novel SSF system is that once higher concentrations of substrate could be handled for fermentation to produce the enzyme, then the volume of liquid used for extracting the cellulase could be reduced to produce higher cellulase-system activity values per unit volume without requiring complex concentration techniques like ultrafiltration.

The results from the pan-bioreactor also show that sodium hydroxide pretreated wheat straw produced higher levels of cellulase activities especially the ß-glucosidase activity. The nature of the substrates may be implicated in this since the steam pretreated and washed substrate contained mostly cellulose which is more recalcitrant for bioconversion compared to the sodium hydroxide pretreated wheat straw which contained both hemicelluloses and cellulose. It has been suggested that the hemicelluloses and amorphous regions of lignocelluloses are utilized before crystalline cellulose during the production of cellulases (Chahal *et al.*, 1992).

5.4.2 Evaluation of Hydrolytic Potential of Cellulase-System: Substrate Enzyme Loading

The profile for the hydrolysis of steam pretreated wheat straw (SWS) with *T. reesei* QMY-1is shown in Fig 5-2. SWS chosen for the hydrolysis experiment since the first results showed that it was not suitable substrate for cellulase-system production. The results show that over 80% of SWS was hydrolyzed into sugars. The profile of the results also showed that while the concentrations of xylose and arabinose remained steady throughout the hydrolysis period, that there was a surge in the formation of glucose after

60 h of hydrolysis with a concomitant decrease in cellobiose, suggesting the transformation of cellobiose units into glucose since the former is a dimer of the latter. A similar profile was obtained from the hydrolysis of the same substrate with *T. reesei* MCG 80. These results were used to set the batch hydrolysis time for the optimization of substrate and enzyme loading for optimum sugar formation from SWS.

Figs 5-3 and 5-4 show the surface plots that illustrate levels of SWS and cellulase loading needed to obtain different concentrations of sugars and a corresponding measure of the hydrolytic potential of the cellulase-systems from both *T. reesei* QMY-1 and MCG 80. Fig 5-3 shows that with less than 5% substrate concentration cellulase loadings over 30 IU/g were needed to achieve over 80% hydrolysis. On the other hand, increasing substrate concentration above 5% resulted in less than 80% hydrolysis at all levels of cellulase loading. The corresponding plot on the total sugar formation also illustrate that more sugars are only formed from increased substrate concentration when the cellulase loading is also increased. The cellulase-system from *T. reesei* QMY-1 also illustrate a similar trend in hydrolysis of SWS and sugar formation. The difference, however, is that even at low substrate concentrations and high cellulase loadings, the cellulase-system from *T. reesei* QMY-1 could not hydrolyze over 70 % SWS.

Overall, the results demonstrate that when steam pretreated wheat straw is chosen for hydrolysis into sugars, the preference will be to use the cellulase-system from *T. reesei* MCG 80 and limit the substrate concentration to less than 5% if over 80% hydrolysis is to be achieved.

5.4.3 Cellulase Production and Cellulose Hydrolysis :A Microstructure Relationship

The microstructures of wheat straw as it passes through the steps of pretreatment, enzyme production and hydrolysis showed clearly the effects of pretreatment on lignocellulose fibers and the extent to which cellulase can hydrolyze these fibers into smaller units to release monomeric sugars. Figs 5-5a, b and c show respectively, unpretreated wheat straw, sodium hydroxide pretreated wheat straw, and cellulase hydrolyzed wheat straw. The pretreated wheat straw showed swollen walls as a result of the swelling action of the NaOH. Fig 5-5c showed broken fibers as a result of the hydrolytic action of the cellulase-system. These structural changes are all important in modifying lignocelluloses for both cellulase production and cellulose hydrolysis and confirms that pretreatment of lignoceluloses is a prerequisite for its hydrolysis (Choudhurry *et al.*, 1984; Gharpuray *et al.*, 1983; Koullas *et al.*, 1992).

5.5 CONCLUSION

A pan-bioreactor was successfully used to increase substrate concentration in the production of cellulase-systems under solid-state fermentation conditions. The advantage associated with this is that the cost of cellulase production could be considerably decreased because of its potential to produce concentrated cellulase-systems with high activities per unit volume. More still, the crude unextracted cellulase has the potential of being utilized for direct hydrolysis without extraction. Steam pretreated and washed wheat straw may not, however, be a good substrate for efficient hydrolysis since it

requires high enzyme loading at low substrate concentration to achieve over 90% hydrolysis.

Table 5-1 Production of cellulase-system on sodium hydroxide pretreated wheat straw (NWS) with *T. reesei* QMY-1 in a pan-bioreactor

Pan-bioreactor	NWS (g/Pan)	Cellulase-system activity (IU/mL)		Cellulase-system activity (IU/g WS)		ßGA/FPA
		FPA	ßGA	FPA	ßGA	
[1]Mesh support	10	7.0	8.0	175	200	1.1
Glass support	10	6.5	4.8	162	120	0.7
Mesh support	20	6.8	4.3	170	108	0.6
Glass support	20	7.7	4.5	193	113	0.6
Mesh support	30	6.4	2.4	160	60	0.4
Glass support	30	4.3	3.1	108	78	0.7
Mesh support	40	6.6	4.1	165	103	0.6
Glass support	40	7.5	4.5	188	113	0.6
Mesh support	50	6.5	2.6	163	65	0.4
Glass support	50	nd	nd	nd	nd	nd

[1] Cellulase-system used for hydrolysis studies; nd = not determined

Table 5-2 Production of cellulase-system on steam pretreated wheat straw (SWS) with *T. reesei* QMY-1 in a pan-bioreactor

Pan-bioreactor	SWS (g/Pan)	Cellulase-system activity (IU/mL)		Cellulase-system activity (IU/g SWS)		ßGA/FPA
		FPA	ßGA	FPA	ßGA	
Mesh support	10	6.5	2.2	123	41	0.3
Glass support	10	5.5	3.1	104	58	0.6
Mesh support	20	6.0	6.7	113	124	1.1
Glass support	20	5.3	6.2	100	115	1.2
Mesh support	30	4.3	1.7	134	53	0.4
Glass support	30	4.1	2.8	77	52	0.7
Mesh support	40	5.2	3.1	98	58	0.6
Glass support	40	4.8	4.0	91	74	0.8
Mesh support	50	4.8	1.5	300	96.0	0.3
Glass support	50	nd	nd	nd	nd	nd

Table 5-3 Production of cellulase-system on sodium hydroxide pretreated wheat straw (NWS) with *T. reesei* MCG 80 in a pan-bioreactor

Pan-bioreactor	NWS (g/Pan)	Cellulase-system activity (IU/mL)		Cellulase-system activity (IU/g WS)		ßGA/FPA
		FPA	ßGA	FPA	ßGA	
Mesh support	10	8.0	6.0	200	150	0.8
Glass support	10	6.5	4.6	162	115	0.7
[1]Mesh support	20	7.3	8.3	183	207	1.1
Glass support	20	4.8	3.0	120	75	0.6
Mesh support	30	7.6	2.3	190	58	0.3
Glass support	30	5.3	0.7	132	17	0.1
Mesh support	40	6.7	2.3	168	58	0.3
Glass support	40	5.5	2.9	138	73	0.5
Mesh support	50	6.9	2.5	173	63	0.4
Glass support	50	nd	nd	nd	nd	nd

[1] Cellulase-system used for hydrolysis studies

Table 5-4 Production of cellulase-system on steam pretreated wheat straw (SWS) with *T. reesei* MCG 80 in a pan-bioreactor

Pan-bioreactor	SWS (g/Pan)	Cellulase-system activity (IU/mL)		Cellulase-system activity (IU/g WS)		ßGA/FPA
		FPA	ßGA	FPA	ßGA	
Mesh support	10	7.3	1.8	137	34	0.2
Glass support	10	6.7	3.4	125	65	0.5
Mesh support	20	7.6	1.2	237	39	0.1
Glass support	20	5.9	2.6	184	84	0.4
Mesh support	30	6.3	2.5	196	78	0.3
Glass support	30	5.4	3.7	168	116	0.7
Mesh support	40	5.7	2.2	179	67	0.4
Glass support	40	4.9	2.8	154	85	0.6
Mesh support	50	5.1	1.2	160	37	0.1
Glass support	50	nd	nd	nd	nd	nd

Fig 5-1 Pan-bioreactor for solid state fermentation

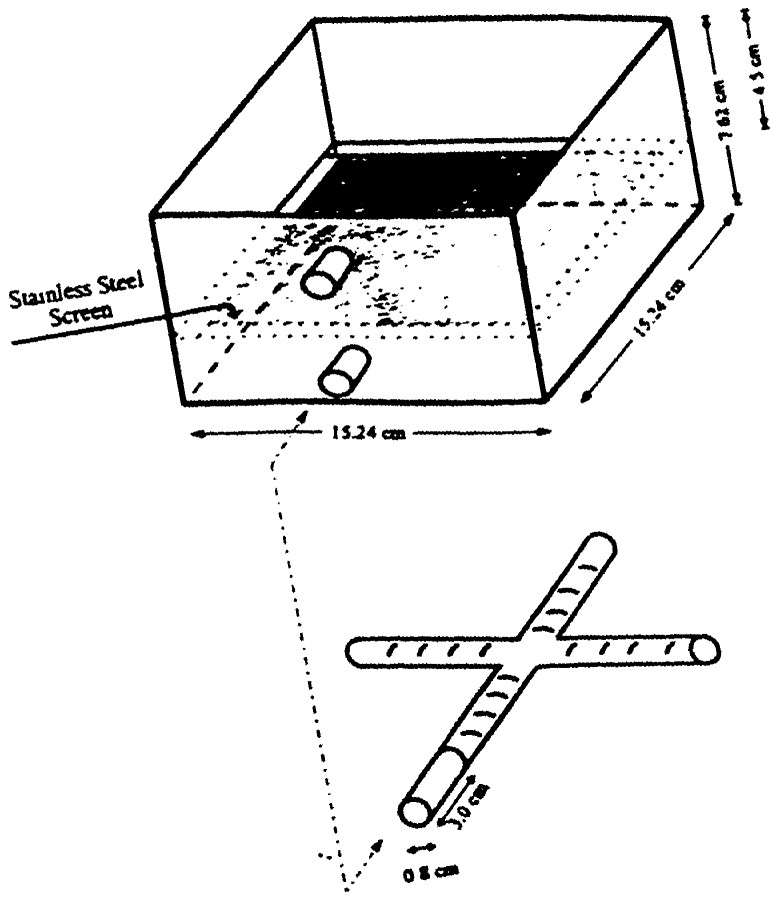
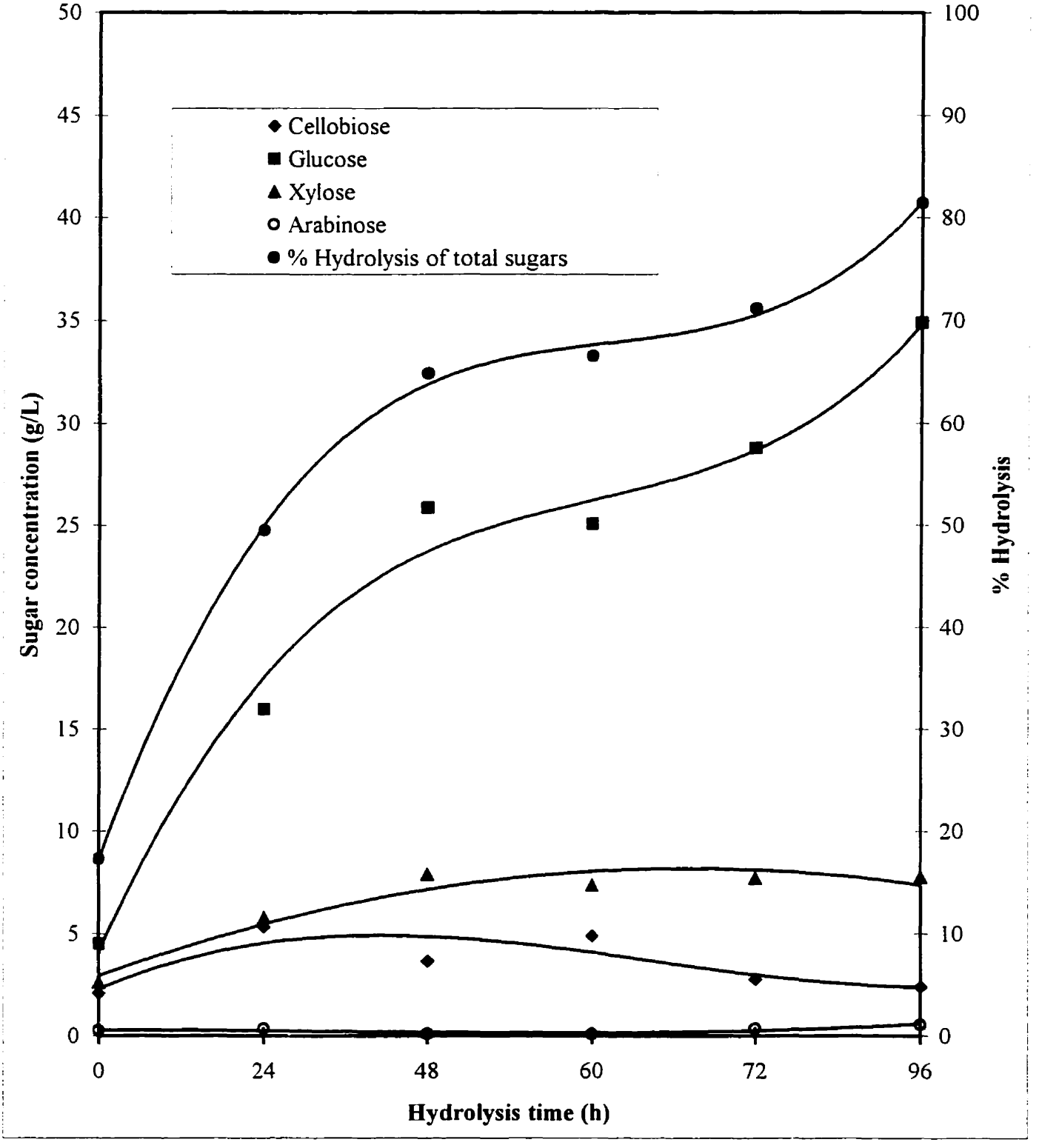


Fig 5-2 Hydrolysis of 5% steam pretreated wheat straw (SWS) with 20 IU/g cellulase-system from *T. reesei* QMY-1



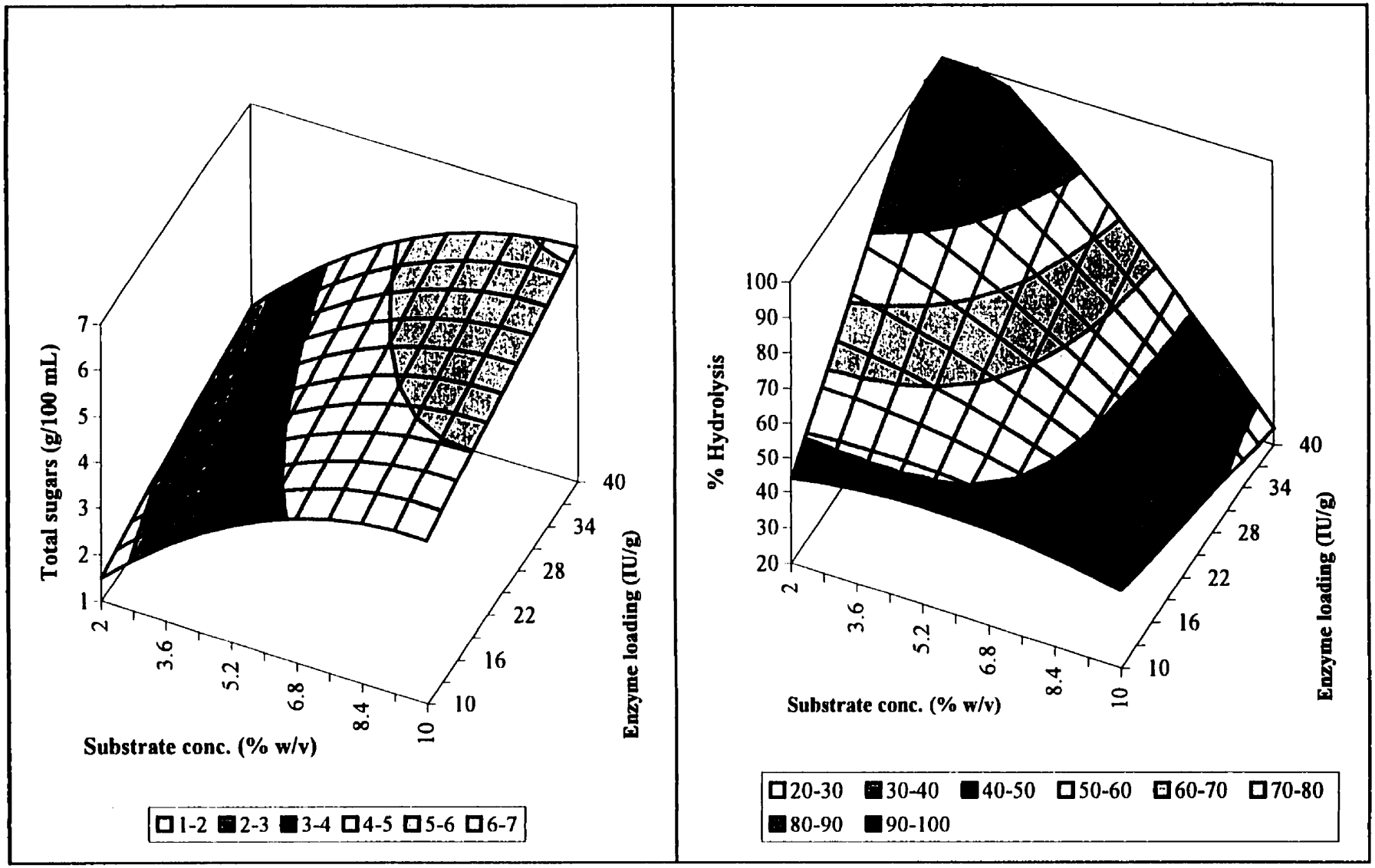


Fig 5-3 Optimization of the hydrolysis of steam treated wheat straw (SWS) with cellulase-system from T. reesei MCG 80

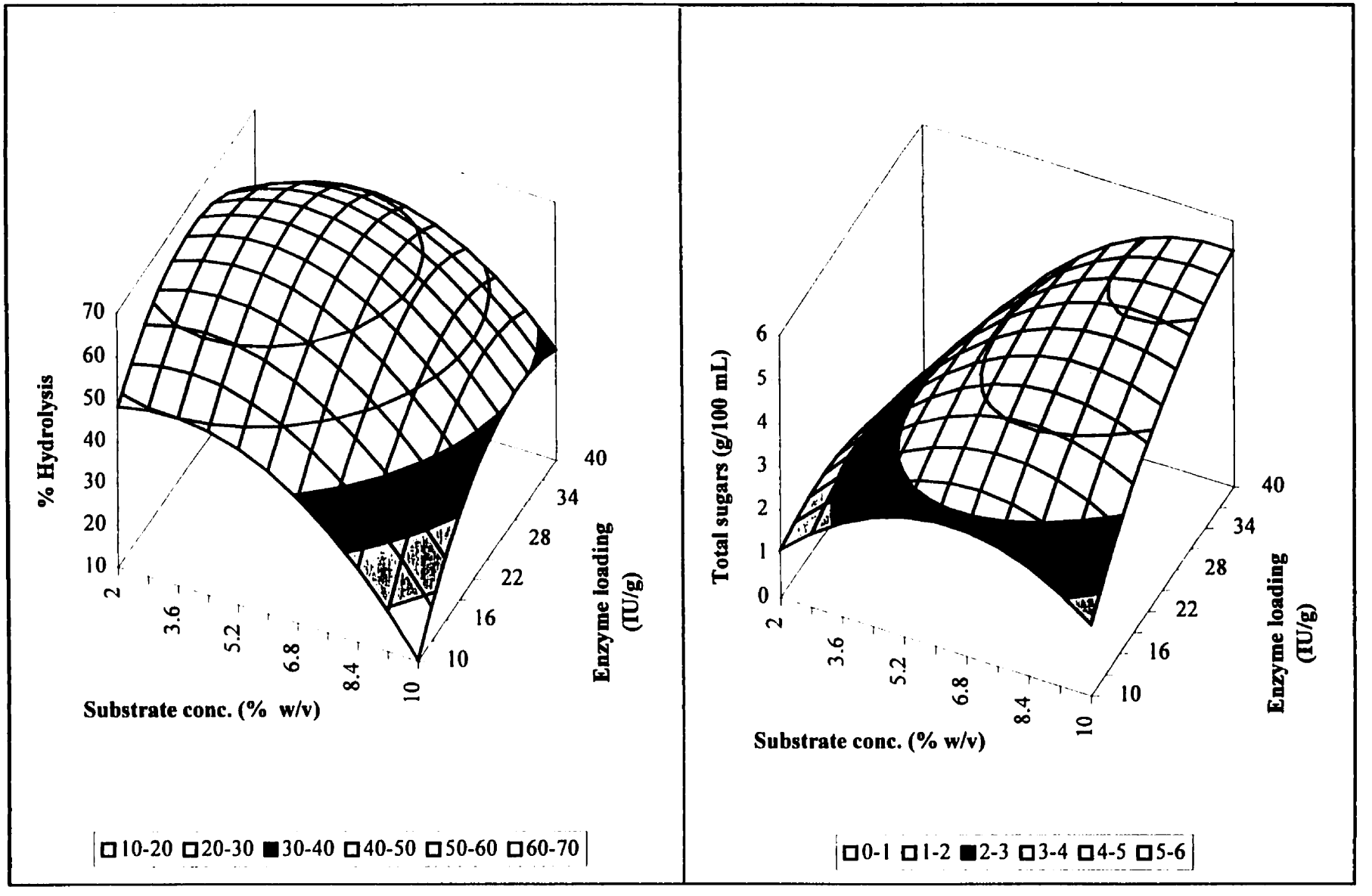
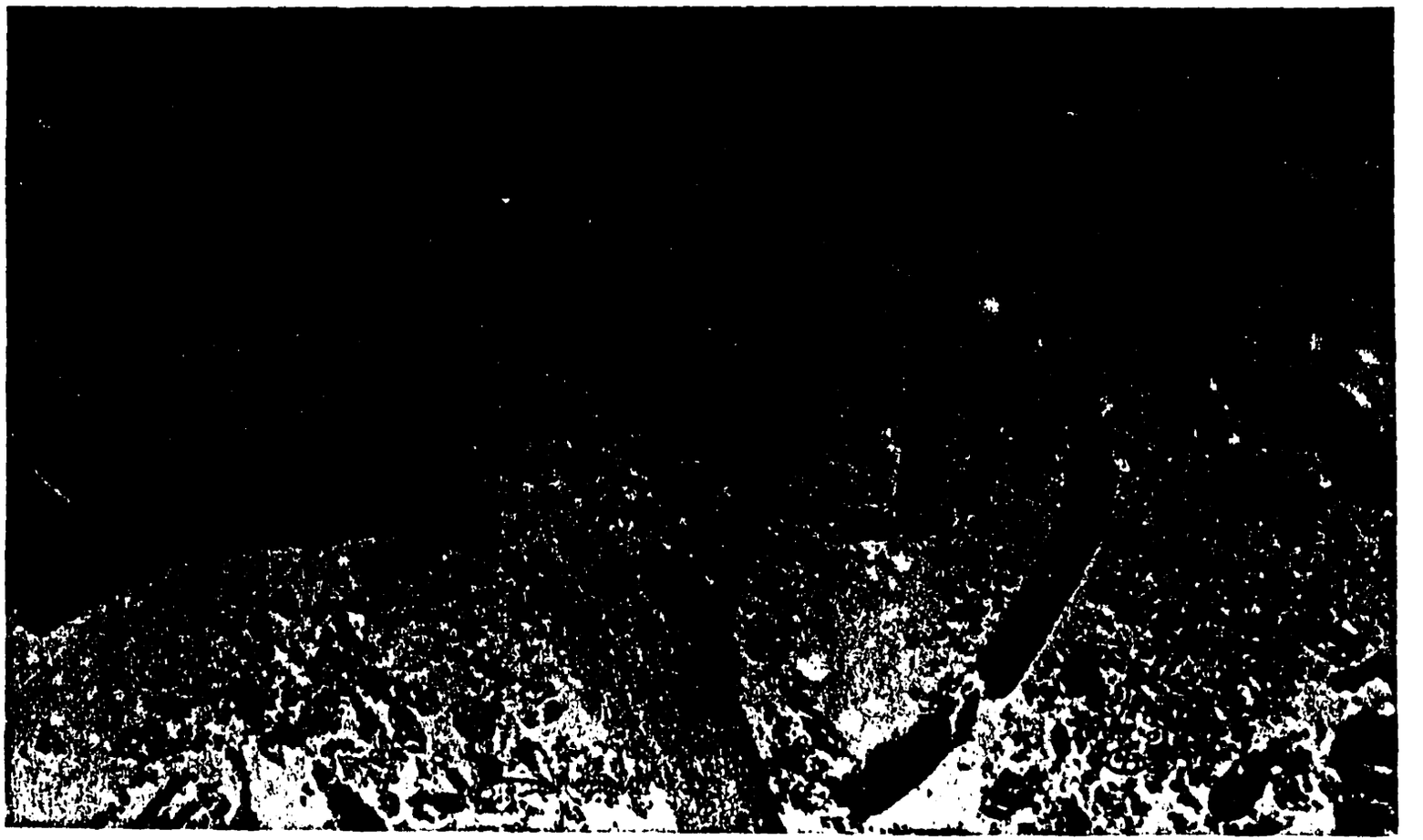


Fig 5-4 Optimization of the hydrolysis of steam treated wheat straw (SWS) with cellulase-system from T. reesei QMY-1

Fig. 5-5a Electron micrograph of Untreated wheat straw (7500 x)

Fig. 5-5b Electron micrograph of fiber modification of wheat straw after pretreatment with 4% sodium hydroxide (7500 x)

Fig. 5-5c Electron micrograph of fiber modification of pretreated wheat straw after hydrolysis with crude extracted cellulase-system from *T. reesei* QMY-1 (7500 x)



CHAPTER 6

PRODUCTION OF CELLULASE-SYSTEMS BY SELECTED MUTANTS OF *TRICHODERMA REESEI* IN SOLID-STATE FERMENTATION AND THEIR HYDROLYTIC POTENTIALS

6.0 Connecting statement

In the previous chapters, the focus was on quantities of cellulase-systems produced from two mutants of *T. reesei*, QMY-1 and MCG 80. This chapter goes further and broadens the scope of cellulase-system producers to include mixed culture systems and then evaluates the cellulase-systems with the particular objective of removing cellobiose accumulation during cellulose hydrolysis with either crude extracted or unextracted cellulase-systems. Comparative studies with commercial cellulases were included.

Note : The text of this chapter has been published as follows :

Awafo, V.A., Chahal, D.S., Simpson, B.K. and Lê G.B.B. 1996. Production of Cellulase-systems by Selected Mutants of *Trichoderma reesei* in Solid-State Fermentation and Their Hydrolytic Potentials. Appl. Biochem. Biotechnol., 57/58 : 461-470.

6.1 ABSTRACT

Three mutants of *Trichoderma reesei* were grown in solid-state fermentation (SSF) in flasks and in a pan bioreactor. Mutant strain MCG 80 proved to be best at producing an optimal cellulase-system using lignocellulosic material (wheat straw) as substrate. This preparation exhibited a beta-glucosidase activity (ßGA) to filter paper activity (FPA) ratio of about 1.0, which is indicative of a high potential for hydrolysis of cellulose. The yields of cellulase-systems and the ratio of ßGA to FPA produced in flasks were comparable to that of the pan bioreactor. The cellulase-system of *T. reesei* MCG 80 having a ratio of ßGA to FPA close to 1.0 gave the most complete (88-95%) hydrolysis of 5% delignified wheat straw (DWS). On the other hand, the cellulase-system of cocultures of *T. reesei* QMY-1 and *Aspergillus phoenicis* failed to produce high hydrolytic yields in spite of having a very high ratio of ßGA to FPA (3.04). This failure was due to the fact that coculture contained the relatively poor quality cellulase-system of the dominant organism, *A. phoenicis.* The resulting fermented wheat straw can be used, as a source of enzyme (unextracted), for hydrolysis of wheat straw and it gives increased yields of reducing sugars compared to analogous extracted enzyme preparations. The hydrolytic potential of two commercial enzymes tested were considerably lower than those of the cellulase-systems produced on wheat straw. It is evident that a complete cellulase-system having ßGA - to FPA- ratio close to 1.0 and high hydrolytic potential can be produced on lignocellulosic feedstocks in SSF

6.2 INTRODUCTION

In the hydrolysis of lignocellulosic feedstocks, the cellulase-system is part of a complex lignocellulolytic enzyme system that hydrolyses glucosidic bonds of crystalline cellulose to glucose. The lignocellulolytic enzyme system comprises ligninases, hemicellulases, pectinases, endo-1,4- ß-glucanases, exo-1,4- ß-glucanases (cellobiohydrolase) and beta-glucosidase. The latter three types of enzymes are collectively referred to as the cellulase-system. Production of an efficient and inexpensive cellulase-system is at the cutting edge in the effort to establish a biotechnological route to complete transformation of cellulose into glucose. A high quality and complete cellulase-system is now realizable using solid-state fermentation (SSF) techniques (Chahal, 1985; Chahal, 1991). Solid-state fermentation is a preferred technique in cellulase production because of the several advantages it has over the submerged or liquid state fermentation method (Hesseltine, 1972; Mudgett, 1986).

The levels of cellulases produced by several cellulolytic microorganisms are comparable with the parent strain, *Trichoderma reesei* QM6a (Saddler, 1986 ; Gomes *et al.*, 1989). Now, several *T. reesei* mutants have been developed for the production of effective and affordable cellulase-systems. The driving force has been to obtain high filter paper activity (FPA) / unit volume and / unit of substrate. The term FPA used in this paper represents the synergistic action of endo- and exo-glucanases. However, the efficiency of hydrolysis of cellulose by a cellulase-system is also governed by the quantities of both the filter paper activity (FPA) and the beta-glucosidase activity (ßGA). More importantly,

a critical factor is the ratio of ßGA to FPA, which should be close to 1.0 (Stockton *et al.*, 1991). This ratio is particularly important in preventing catabolic repression of the endoglucanase and cellobiohydrolase by ensuring that cellobiose does not accumulate during the course of the reaction. Therefore, a cellulase-system having ßGA to FPA ratio close to 1.0 is called a "complete" cellulase-system since it establishes a threshold enzyme ratio for the elimination of accumulated cellobiose during hydrolysis of cellobiose. It has been estimated that in cellulose hydrolysis (10-400 g/L), a cellobiose concentration of 0.01-0.1 g/L almost completely inhibits cellobiohydrolase (Klyosov,1980).

Although the literature abounds with filter paper and beta-glucosidase production values (Esterbauer *et al.*, 1991; Panda *et al.*, 1983) and a few cellulase quality assessment studies (Morriset and Khan, 1984; Breuil *et al.*, 1992), very little work is available on the hydrolytic potential of cellulase-systems produced by different mutants of *T. reesei* using varied conditions and practical substrates. Hydrolytic potential is defined as the extent to which a given substrate can be digested by a set loading of enzyme over a defined period of time and reaction conditions.

Economic production of fuel ethanol from cellulose calls for the need to compare different cellulase producing micro-organisms in terms of their ability to produce sufficient quantities of the complete cellulase-system to efficiently hydrolyse cellulose to glucose.

T. reesei occupies a dominant role in the continued search for an efficient cellulase-system. This study assesses different mutants evolving from *T. reesei* QM6a (the original

strain) for the efficiencies of their cellulase-systems.

In the present study, we report the biochemical characteristics and hydrolytic potentials of cellulase-systems of selected mutants of *T. reesei* when cultured individually and also in cocultures with *Aspergillus phoenicis* (a ßGA producer) in SSF in flasks and in a specially designed pan bioreactor (chapter 5).

6.3 MATERIALS AND METHODS

6.3.1 Microorganisms

Trichoderma reesei mutants QMY-1 (NRRL 18760), QM 9414 (NRRL 6165) and MCG 80 (NRRL 12368) were cultivated individually and in cocultures with *Aspergillus phoenicis* (NRRL 1956) in Mandels medium (Mandels and Weber, 1969). The genealogy of the *Trichoderma reesei* mutants is as follows : QMY-1 is a direct descendant of QM 9414 obtained in our laboratory by Dr. D.S. Chahal while MCG 80 is a direct descendant of *T. reesei* Rut. C-30. *Aspergillus phoenicis* was selected for its ability to produce high levels of beta-glucosidase (Sternberg *et al.*, 1977; Srivastava *et al.*, 1981). *Trichoderma reesei* MCG 80, QM 9414 and *A. phoenicis* were obtained from Dr. J. L. Swezey, ARS Patent Culture Collection, USDA, Illinois, USA. All the test organisms were maintained on agar in Petri plates with Mandels medium having delignified wheat straw as carbon source.

6.3.2 Commercial Enzymes

Commercial cellulase sources were tested for their hydrolytic potential and compared with cellulase-systems obtained from *T. reesei* mutants produced under SSF conditions. The commercial cellulases from Genecor and Iogen were supplied by Trichromatic Techno-Chem, Quebec, Canada.

6.3.3 Substrate and Enzyme Production

Wheat straw has the following approximate chemical composition : Cellulose (39%), hemicelluloses (36%), lignin (10%) and silica (6%) (Staniforth, 1979).

Ground wheat straw (20 mesh) was treated with 4% NaOH (w/w) to a moisture content of 70% (dwb) and left overnight. The treated substrate without washing was autoclaved at 121 °C for 30 minutes. Twenty grams, on dry weight basis (dwb), of the treated and autoclaved wheat straw (WS) were used for each pan bioreactor while each Erlenmeyer flask contained 4 g (dwb) of WS. The nutrient elements of Mandels medium were calculated by weight of the polysaccharide (cellulose and hemicelluloses) content of WS and added to the substrate and the final pH adjusted to 6.5. Five mL and 25 mL of 36h grown mycelium of the mutants were inoculated, respectively, on the surface of each flask and pan. The moisture content of the substrate after inoculation was about 75% (dwb). The cultures were held in a humidified incubator (Norco 4100) and samples were harvested after 20 days of cultivation.

6.3.4 Enzyme Extraction and Analysis

Water was added to the fermented substrate to a final volume of 100 mL. The substrate was agitated in a rotary shaker at 200 rpm for 30 minutes, then centrifuged at 10 000 rpm for 30 minutes. The supernatant was used for enzyme analyses and cellulose hydrolysis. FPA and ßGA were determined by the method of Mandels *et al.*. (1976) approved by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987).

6.3.5 Hydrolysis of Cellulose

To examine the hydrolytic potential of cellulase-systems, hydrolysis was performed on delignified wheat straw (DWS) using cellulase-systems obtained from wheat straw without urea in the cultivation medium. Wheat straw was delignified by the method of Toyama and Ogawa (1972). The extracted cellulase-system was supplied at the rate of 20 IU FPA/ g DWS. Crude unextracted enzyme (CUC) as found in the resulting fermented wheat straw was also used for the hydrolysis of DWS. The CUC is that portion of the resulting fermented wheat straw in SSF that was not subjected to extraction as explained above. The quantities of CUC to supply 20 IU FPA/ g DWS were calculated based on the filter paper activities of the extracted enzyme per g fermented substrate. The required enzyme units were added to 5% and 10% DWS in citrate buffer (0.1M, pH 4.8) in Erlenmeyer flasks and incubated at 45 °C for 60 h with agitation at 200 rpm.

6.3.6 Analysis of Hydrolysates

Samples were quantitatively analysed for dominating sugars such as glucose, cellobiose, xylose and arabinose with a Beckman 344 HPLC system, degassed deionised water as mobile phase, an Aminex HPX-87P column heated to 85 °C, and an Altex 156 Refractive Index Detector. In the hydrolysate, glucose and cellobiose (a dimer of glucose) are products of cellulose while xylose and arabinose (pentoses) are from the residual hemicelluloses in the DWS.

6.4 RESULTS AND DISCUSSION

6.4.1 Comparison of cellulase-systems produced from *T. reesei* mutants and their cocultures with *A. phoenicis*.

Table 6-1 shows the comparison of characteristics of cellulase-systems produced from three mutants of *T. reesei* : QM 9414, QMY-1 and MCG 80 alone and in cocultures with *A. phoenicis* in SSF for 20 days with Mandels medium with or without urea. Urea in the medium not only supplies nitrogen, but also offsets drastic pH drop during growth in SSF. When the *T. reesei* mutants were cultivated individually in Mandels medium without urea, the MCG 80 appeared to have an edge in both FPA and ßGA over all the mutants and had a ratio of ßGA to FPA of 1.0. The QMY-1, which is a good producer of cellulase-system in SSF with ßGA to FPA ratio close to 1.0 (Chahal, 1991), failed to achieve this ratio when urea was eliminated from the medium. The QM 9414 also

produced low yields of cellulase-system with low ratio of ßGA to FPA in the medium without urea. The addition of urea, however, improved the cellulase-system of *T. reesei* QMY-1 in pan bioreactor and it was comparable to that of MCG 80 except that the ratio of ßGA to FPA was a little low (0.71).

Co-culturing the *T. reesei* mutants with *A. phoenicis* in SSF did not improve cellulase-system production but instead had a detrimental effect on the FPA yields. The apparent growth of the cocultures indicated that *A. phoenicis* outgrew the *T. reesei* mutants in SSF. *A. phoenicis* alone produced low FPA and high ßGA, therefore, its domination in cocultures resulted in the production of low FPA and high ßGA. Thus a ratio of ßGA to FPA more than 3.0 was obtained.

6.4.2 Hydrolytic potential of different Cellulase- Systems

For this experiment, the cellulase-system of MCG 80 was selected for hydrolysis of DWS because it had the desired ratio of ßGA to FPA of 1.0. The other cellulase-systems were selected because of the following qualities: the cellulase-system of QMY-1 produced on medium without urea had low ratio of ßGA to FPA and that of cocultures of QMY-1 and *A. phoenicis* had very high ratio of ßGA to FPA (3.41). The commercial enzymes were tested because they showed low ratios of ßGA to FPA (0.32 and 0.60) and it is assumed that these enzymes were produced by submerged (liquid) state fermentation.

These commercial enzymes when tested in our laboratory showed very high FPA and ßGA per mL. However, the ratio of ßGA to FPA for the Genencor cellulase was 0.32

whereas that of Iogen cellulase was 0.6 (Table 6-2), which are low with respect to hydrolytic potential and from the point of view of our definition of a "complete" cellulase-system.

The data presented in Table 6-2 clearly indicate that the cellulase-system of MCG 80 gave the highest hydrolytic potential (88-95%) whereas other enzyme systems gave low hydrolytic potentials (varying from 33.5 to 51.8%). The low hydrolytic potential of QMY-1 reported here is probably due to the low ratio of ßGA to FPA (0.34) produced in a medium without urea. In an earlier study, when urea was added to the cultivation medium of QMY-1, the ratio of ßGA to FPA was close to 1.0 and over 90% hydrolytic potential was recorded (Chahal, 1985).

It is worth noting that the CUC gave higher hydrolytic potential in all the three cases (Table 6-2). The higher hydrolytic potential could be due to the fact that the use of CUC could have been an additional source of cellulose and cellulase. The use of fermented WS as such (CUC) has two major advantages: (i) it would eliminate the need to extract enzyme and (ii) unutilized cellulose in the production phase will have a second chance for conversion into glucose. Both factors contribute to reducing the cost of ethanol production from lignocellulosic feedstocks.

The composition of hydrolysates obtained with these cellulase-systems indicated that in the case of MCG 80 when the ratio of ßGA to FPA was 1.0, the cellobiose concentration was low and therefore did not inhibit the hydrolysis (Figs. 6-1, 6-2 and 6-3). The hydrolytic efficiency of CUC of *T. ressei* MCG 80 went up as high as 95% even though

the cellobiose concentration in the hydrolysate was as high as 0.8 g/L in 5% delignified wheat straw digestions (Fig. 6-1). This calls to question the earlier suggestion of Klyosov (1980) that cellobiose concentrations of 0.01 - 0.1g/L completely inhibit cellobiohydrolase in cellulose hydrolysis concentration of 10 - 400 g/L.

At 5% DWS, CUC from MCG 80 produced less cellobiose in the hydrolysate compared to its analogous extracted enzyme (Fig. 6-1). This was probably as a result of availability of adsorbed enzyme in the CUC during hydrolysis. The cellobiose content was higher in the hydrolysates of the QMY-1 samples compared to the MCG 80 samples (Figs. 6-1 and 6-2). The plausible explanation is that MCG 80 had higher ßGA than QMY-1 (Table 6-2) since beta-glucosidase has a critical role in preventing cellobiose accumulation during hydrolysis of cellulose.

In the case of the hydrolysate from the cellulase-system of coculture of QMY-1 with *A. phoenicis* (Fig. 6-3), at a ratio of ßGA to FPA greater than 1.0, no cellobiose in the hydrolysate was noticed. Even with no inhibition of cellulases because of the absence of cellobiose, the hydrolytic potential was still very low, although the quantity of FPA loading was 20 IU/g in all the cases. Low hydrolytic potentials in the cellulase-systems of cocultures and commercial enzymes could be attributed to the fact of the poor quality of cellulase-systems produced under certain cultural conditions as reported earlier (Chahal, 1991). It may, therefore, be noted that equal enzyme loadings from different sources could result in different hydrolytic potentials (Table 6-2) suggesting the presence of a quality attribute not apparent when enzyme loadings are based on FPA alone.

High sugar concentration in the hydrolysate will also contribute to lower the overall cost of ethanol production. Thus, an efficient cellulase-system should have the capability to hydrolyse a high concentration of cellulose. When the substrate concentration for hydrolysis was increased from 5% to 10%, there was an increase in total sugar concentration in the hydrolysate in all the cases (Table 6-3). Further examination of the data indicated, however, that there was a decrease in percentage of hydrolysis with increase in substrate concentration. This decrease was lowest (13%) in the case of QMY-1 compared to that of other cellulase-systems (about 25%). It is evident from the data (Table 6-3) that some cellulase-systems like that of QMY-1 can tolerate higher substrate concentration than others (MCG 80) at the same enzyme loading of 20 IU/g.

In fact, when the cellulase-system of QMY-1 had the ratio of ßGA to FPA about 1.0, it hydrolysed 10% DWS to the extent of 89 - 100% (Chahal, 1985). The cellulase-system of the coculture was the least tolerant to the high substrate concentration. This again might be due to the poor quality of the cellulase-system as already explained above. It appears that a cellulase-system should also have high tolerance to substrate concentration for a viable hydrolysis process.

6.5 CONCLUSION

It is evident from the study that a complete cellulase-system having ratio of ßGA to FPA close to 1.0 and high hydrolytic potential can be produced with suitable mutants of *T. reesei* in a pan bioreactor in SSF. Such a complete cellulase-system does not require an

external source of beta-glucosidase for cellulose hydrolysis. Co-culturing of *T. reesei* mutants with *A. phoenicis* increased yields of ßGA in the cellulase-system but proved to be detrimental to the FPA production in SSF.

Table 6-1 Comparison of cellulase-systems produced with mutants of *Trichoderma reesei*

Fermentation system	FPA			βGA (IU/mL)	βGA/FPA
	IU/mL	IU/g substrate	IU/g cellulose		
QM 9414, no urea in flask	3.75	94	235	1.11	0.30
QMY-1, no urea in flask	3.58	90	224	1.33	0.37
MCG 80, no urea in flask	5.33	133	333	5.33	1.00
QM 9414 + AP, no urea in flask	1.01	25	63	3.78	3.70
QMY-1 + AP, no urea in flask	1.08	27	68	2.80	2.60
MCG 80 + AP, no urea in flask	1.02	26	64	4.10	4.00
Aspergillus phoenicis (AP)	1.02	26	64	3.24	3.17
QMY-1 + 0.1% urea in pan	9.55	129	322	6.80	0.71
QMY-1, no urea in pan	6.35	86	214	2.15	0.34
MCG 80, no urea in pan	9.25	125	312	9.55	1.03
QMY-1 + AP, no urea in pan	2.04	28	69	6.96	3.41
Genencor	354.57	-	-	114.71	0.32
Iogen	329.26	-	-	197.46	0.6

Table 6-2 Comparison of Hydrolytic Potential of Different cellulase-systems

Cultural condition	Type of fermentation	FPA IU/mL	βGA IU/mL	Ratio βGA/FPA	Hydrolytic Potential (%)[1]	
					Extracted enzyme (CEC)	Unextracted enzyme (CUC)
T. reesei QMY-1 without urea	SSF in pan bioreactor	6.34	2.15	0.34	51.80	69.10
T. reesei MCG 80 without urea	SSF in pan bioreactor	9.25	9.55	1.03	88.00	95.30
T. reesei QMY-1 + AP without urea	SSF in pan bioreactor	2.04	6.96	3.41	33.50	49.80
Genencor	Not known	354.60	114.71	0.32	44.20	ND
Iogen	Not known	329.26	197.46	0.60	41.30	ND

[1] Hydrolytic potential = 100 (Rs/Wt), where Rs is total reducing sugars and Wt is weight of DWS

ND = Not determined.

Table 6-3 Effect of different Cellulase-systems and Substrate Concentration on the Hydrolysis of Delignified Wheat Straw (DWS)

Cellulase-system [1]	5% DWS		10% DWS		Decrease in hydrolysis with increase in substrate conc. (%)
	Total sugars (g/L)	Hydrolysis (%)	Total sugars (g/L)	Hydrolysis (%)	
T. reesei MCG 80	48.4	88	74.0	67	24
T. reesei QMY-1	28.5	52	49.0	45	13
T. reesei QMY-1 + *A. phoenicis*	18.7	34	27.0	25	25

[1] Extracted cellulase was used for hydrolysis.

Total sugars = {cellobiose + glucose + xylose + arabinose }

Fig. 6-1 Hydrolysis of delignified wheat straw (DWS), with cellulase-system from *T. reesei* MCG 80 and commercial enzymes, after 60 h incubation

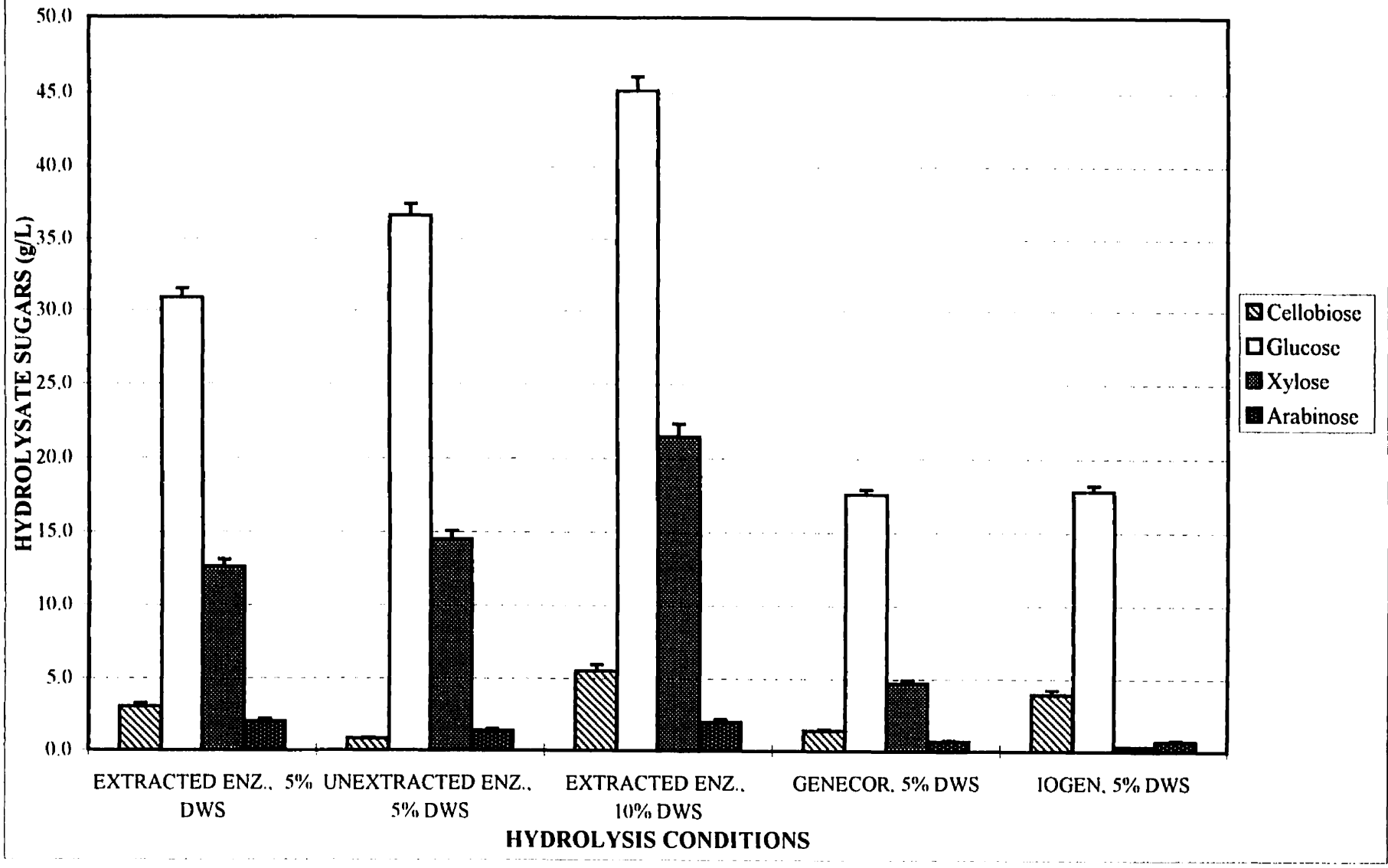


Fig. 6-2 Hydrolysis of delignified wheat straw (DWS), with cellulase-system from *T. reesei* QMY-1 and commercial enzymes, after 60 h incubation

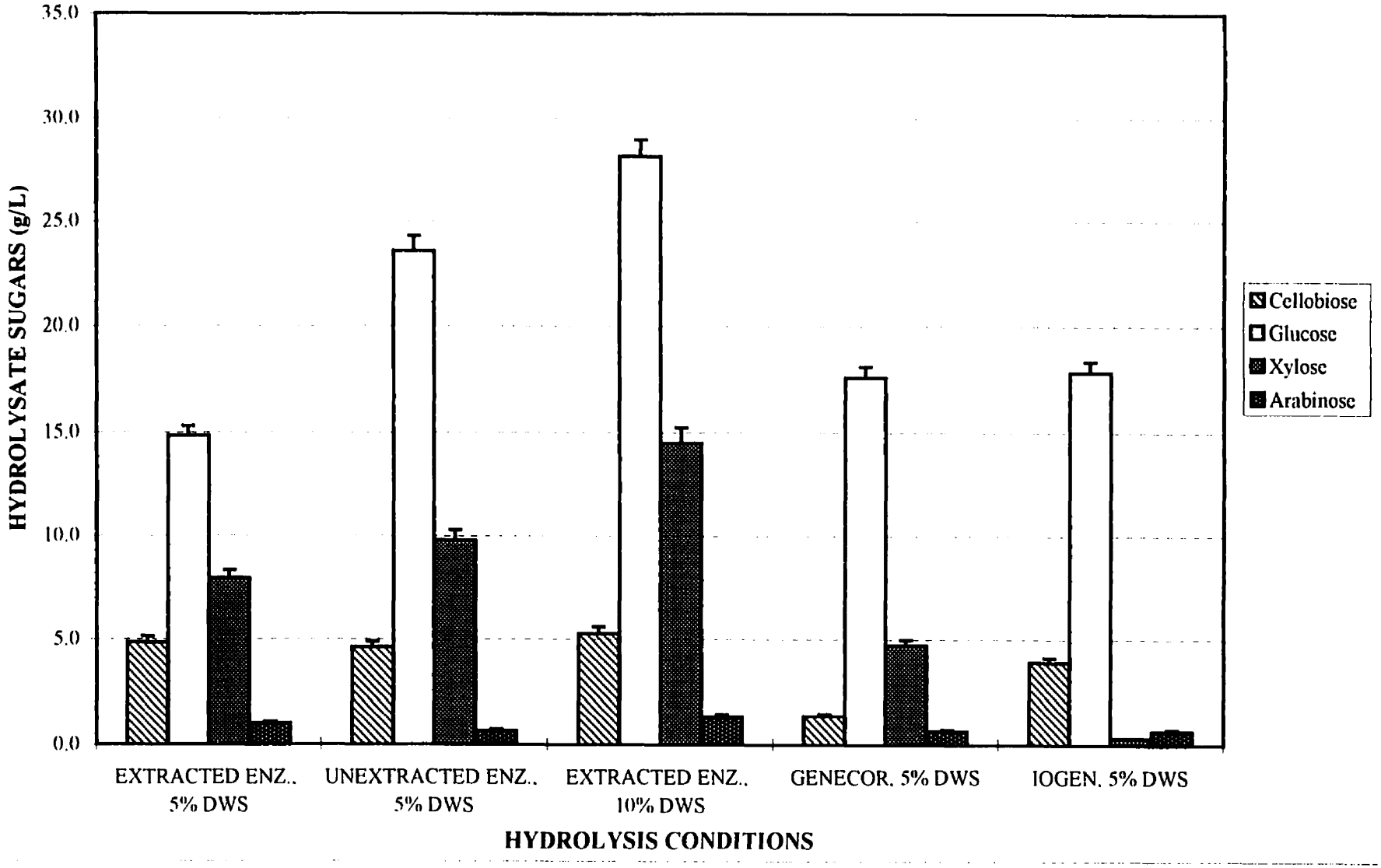
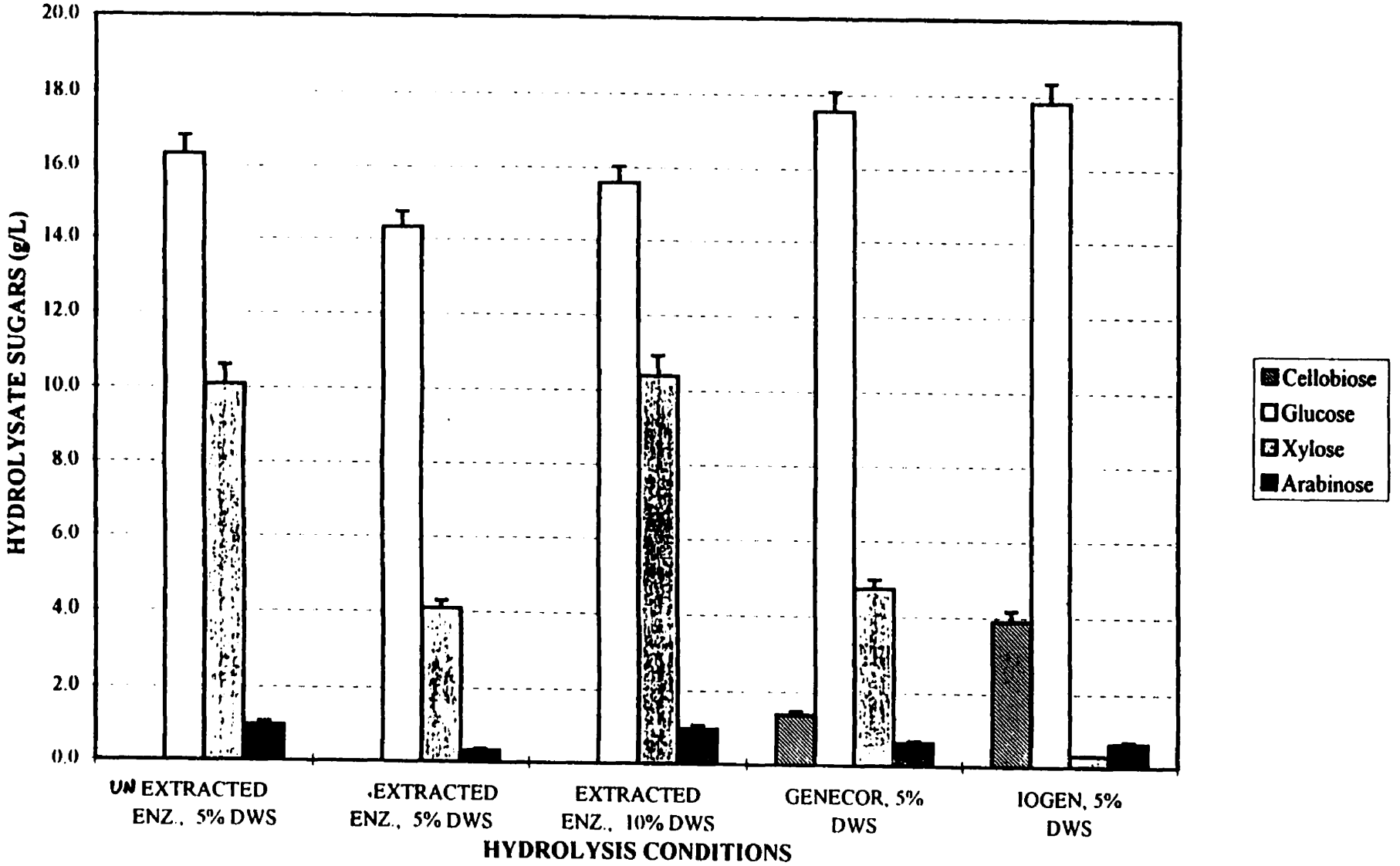


Fig. 6-3 Hydrolysis of delignified wheat straw (DWS) with cellulase-system from coculture of *T. reesei* QMY-1 and *A. phoenicis*, and commercial enzymes, after 60 h incubation



CHAPTER 7

PRODUCTION OF ETHANOL THROUGH ENZYMATIC HYDROLYSIS OF DELIGNIFIED WHEAT STRAW

7.0 Connecting statement

A good cellulase-system with high hydrolytic potential answers the challenge for ethanol production from lignocelluloses if the processes for cellulase-system production and cellulose hydrolysis are linked to a fermentation process to produce ethanol. This chapter explores these three processes together but as separate unit operations. Information from this study will be compared to another (chapter 8) in which the hydrolysis and fermentation processes are treated as one unit operation. It is only when process parameters are well defined that fuel ethanol production from lignocelluloses can be commercialized.

Note : This chapter is the text of a manuscript submitted for publication as follows :

Awafo, V.A., Chahal, D.S., Simpson B.K. 1997. Production of ethanol through enzymatic hydrolysis of delignified wheat straw. J.Food Biochem.

Contribution of Co-authors : Chahal, D.S., Simpson B.K (Thesis co-supervisors)

7.1 ABSTRACT

Wheat straw can be efficiently utilized both as substrate for the production of a complete cellulase-system with *T reseei* QMY-1 and for the hydrolysis of its holocellulose portion into simple sugars for their fermentation into ethanol. When sodium hydroxide and urea were optimized for a complete cellulase-system production, the highest filter paper activity, 8.2 IU/mL (205.7 IU/g WS), was obtained when 4% sodium hydroxide and no urea were used for the pretreatment of wheat straw. However, the presence of urea at a concentration level of 0.015% (w/w), favored the production of ß-glucosidase up to 7 IU/mL (170 IU/g WS). The quality of the cellulase-system was good enough to produce over 85% hydrolysis of 50 g/L delignified wheat straw. The unextracted cellulase-system even performed better than some commercial cellulases from Genencor and Iogen. Out of three hexose- and pentose- fermenting yeasts examined, *P. stipitis* was the best at converting the delignified wheat straw hydrolysate into ethanol (0.51 g/g DWS, 99%)

7.2 INTRODUCTION

The world wide annual production of wheat is estimated to be over 578 million metric tons (USDA, 1996) and this yields over 867 million metric tons of straw. This figure translates into about 33% of the total world annual grain and cereal crop residues and by far the largest and followed at a distant second by rice straw (22%). The ratio of wheat straw to grain is estimated between 1:1 and 1:5 (Ishaque and Chahal, 1991 ; Zakia Bano and Rajarathnam., 1988). In Canada, wheat production for the same period was estimated to be 27.5 million metric tons giving an equivalent amount of about 41 million metric tons of wheat straw. Depending on the agricultural practices of the region of wheat production, the

wheat straw is either collected as a low value feed for ruminant animals because of their recalcitrant nature, burnt or left on the fields. Wheat straw, however, can also be routed for the production of a high quality cellulase-system with ratio of b-glucosidase activity to filter paper activity close to one for effective and efficient hydrolysis of pretreated wheat straw and other cellulosic materials. Pretreatment of wheat straw is a pre-requisite for exposing its holocellulose (cellulose and hemicelluloses) content for enzymatic hydrolysis. Wheat straw holocellulose can be obtained by delignification (Toyama and Ogawa, 1972).

In the native state, wheat straw has an approximate chemical composition of 75% holocellulose (cellulose and hemicelluloses), 10% lignin and 6% silica (Staniforth, 1979) and when delignified, it contains about 90% holocellulose. Although delignified wheat straw (DWS) will be low in lignin, it will still have some hemicelluloses mixed with cellulose fibres which on hydrolysis will produce a hexose and pentose-rich hydrolysate. The model for cellulose hydrolysis involves diffusion of the cellulase system from the bulk fluid to the solid substrate and adsorption of the cellulase onto available sites of the cellulose particle. There is then formation of cellulase-cellulose reactive complex followed by hydrolysis of the glycosidic bond of the cellulose polymer. The hydrolysis products, namely, hexoses and pentoses and other low molecular weight compounds diffuse from the active site to the bulk fluid.

Glucose is the main hexose component which can be used as chemical feedstock for the manufacture of ethanol, acetone, butanol, 2,3-butanediol and other chemicals. The xylose component of the delignified wheat straw can also be transformed into ethanol, xylitol and other low molecular weight compounds. Traditionally, the use of lignocellulose hydrolysates as substrates for ethanol production may cause problems due to their complex composition as a consequence of the substrate type and its pretreatment. This could result in substances that may exert inhibitory effects on the fermentation of sugars to ethanol and other compounds (Olsson and Hahn-Hägerdal, 1992). Several lignocellulosic hydrolysates

have been examined for ethanol production, i.e., spent sulfite liquor from wood and steam-pretreated wood (*salix caprea*) (Olsson and Hahn-Hägerdal, 1992), and sugarcane bagasse hydrolysate (Roberto *et al.*, 1991).

The hydrolysis products can be obtained separately and then fermented into ethanol where the two processes proceed in different vessels, or the fermentation is allowed to proceed at the same time that the hydrolysis products are being formed in the same vessel. In the former case, optimum conditions can prevail for both processes of hydrolysis and fermentation which invariably are always different for each process. Attempts to obtain thermo-tolerant yeast strains are still evasive especially when it comes to naturally occurring mixed hydrolysates. For instance the cellulase-system of *T. reesei* hydrolyses cellulose at high temperatures of 40-50°C, and yet fermentation with excellent yeast strains always limit the temperature to less than 40°C. In our previous study (Awafo *et al.*, 1996), very high sugar concentrations up to 48 g/L and 74 g/L have been obtained respectively from 50 g/L and 100 g/L substrates, thus, offsetting the problem of hydrolysis products having inhibitory effects on the cellulase-system used for the hydrolysis. More still, medium formulation for the mixed sugars of lignocellulose hydrolysates can be proscribed to give optimum fermentation products (ethanol) and information obtained from the composition of the hydrolysates helps in the choice of fermenting microorganisms especially when new substrates are involved.

The choice of microorganisms to ferment mixed hexoses and pentoses resulting from lignocellulose hydrolysates has caused problems in the past. Slapack *et al.*, (1983) reported that *Candida tropicalis* consumed the produced ethanol before the onset of xylose utilization. Diauxie has been encountered in *Pachysolen tannophilus* fermentation of xylose or cellobiose in the presence of glucose resulting in prolonged lag periods (Detroy *et al.*, 1982; Kilian *et al.*, 1983). This latter phenomenon was also observed with *Candida shehatae* in artificially mixed sugar fermentation (Du Preez *et al.*, 1986) where complete

diauxie was observed.

The presence of ethanol in fermentation media has been demonstrated to severely inhibit yeasts. It has been estimated that cell growth stops at relatively low ethanol concentrations and fermentation stops at relatively higher ones (Stokes, 1970). Cell growth inhibition by ethanol is noncompetitive and may be linear (Holzberg *et al.*, 1967) or an exponential function of ethanol concentration (Aiba *et al.*, 1968).

Simultaneous hydrolysis and fermentation also has its own advantages prominent among which include reduction in cost of production since one vessel is involved and less time and labor will be required to complete the two processes.

In a study comparing fermentation of hydrolysate from enzymatically hydrolyzed steam-pretreated willow with different microorganisms, Olsson and Hahn-Hägerdal (1993) had shown that the yeast *S. cerevisiae* and the bacterium *Zymomonas mobilis* were the two most effective microorganisms that gave a yield of 0.46 g ethanol/g glucose in 48 h at 30°C.

The present paper reports an optimization model for the production of a complete cellulase-system from wheat straw, the hydrolysis of delignified wheat straw with same cellulase-system and fermentation of its hydrolysate, containing mixtures of hexose and pentose, with various yeasts known to ferment mixtures of glucose and xylose.

7.3 MATERIALS AND METHODS

7.3.1 Materials

Ground wheat straw (WS) and delignified wheat straw (DWS) were used as substrates for the cellulase-system production and hydrolysis, respectively. Commercial cellulases from

Iogen and Genencor were supplied by Trichromatic Techno-Chem, Québec, Canada.

7.3.2 Cellulase Production - Experimental design

Since pretreatment of lignocelluloses is a pre-requisite for good cellulase-system production, an experimental model was designed to optimize the concentration of NaOH for the pretreatment of WS alongside with urea. The presence of urea in the fermentation medium can have drastic effect on the pH during fermentation and this is particularly of interest in solid-state fermentation for cellulase-system production where the fermentation lasts for a considerable period of time with no pH controls.

A second order central composite design was used to study the effect of NaOH concentration (2-6%) and urea (0.0075-0.0225%) on cellulase-system activity expressed as Filter Paper Activity (FPA) and b-glucosidase activity (ßGA). A complete cellulase-system is one that has the ratio of ßGA to FPA close to one. This ratio is important to ensure there is no feedback inhibition of cellobiose. Five replicates at the central points were performed to determine standard error and there were four kernel points and four star points with a = 1.4142.

The independent variables were coded as:

$$N = (n - 4)/2 \quad (1)$$

$$U = (u - 0.015)/ 0.0075 \quad (2)$$

Where n = NaOH concentration (%)

u = Urea concentration (%)

The equation for the model takes the form

$$FPA = X_0 + X_1(N) + X_2(U) + X_{11}(N^2) + X_{22}(U^2) + X_{12}(U)(N) \quad (3)$$

where FPA = Filter Paper Activity

A similar model function was used for ß-glucosidase activity. All values are mean values of two experimental data points.

7.3.3 Solid-State Fermentation (SSF)

Each experiment was set up in a 100 mL Erlenmeyer flask containing 4 g (dwb) pretreated wheat straw. The pretreated wheat straw was fortified with 4 mL Mandels medium (Mandels and Weber, 1969) based on the carbohydrate content of the wheat straw and to give a final moisture content of 75%. After sterilization at 121°C for 30 min., the substrate was inoculated with the culture of *T. reesei* QMY-1 and incubated in a humidified incubator at 30°C for 21 days. Harvested samples were analyzed for their FPA and ßGA.

7.3.4 Enzyme assays

Distilled water was added to each fermented flask based on the extraction ratio of 4 g dry weight basis per 100 mL water. Tween 80 was added at 0.1% in the slurry. The mixture was shaken on a rotary shaker for 30 min. and then centrifuged at 10, 000 rpm for 30 min. The supernatant was used for the individual enzyme assay and only the cellulase-system from the experiment that produced the highest FPA was used for the hydrolysis of DWS. The FPA and ßGA of the crude extracts and commercial enzymes were determined as approved by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987).

7.3.5 Hydrolysis of wheat straw

Wheat straw was delignified according to the method of Toyama and Ogawa (1972). The delignified wheat straw was then subjected to hydrolysis based on 5% substrate concentration with 20 IU FPA/g substrate at 45°C. Four different cellulase sources were used for the hydrolysis; crude extracted enzyme (CEE), crude unextracted enzyme (CUE), Iogen commercial enzyme, and Genencor commercial enzyme. Crude unextracted enzyme (CUE) is that portion of the fermented solid substrate that has not been subjected to extraction. Residual substrate, including unutilized cellulose, is part of the crude unextracted cellulase-system. After 72 h of hydrolysis, the hydrolysate was centrifuged at 11 000 g, at 4°C to obtain the supernatant. The supernatant was heated for 3 min., and passed through 0.45μ m filters. Individual soluble sugars were determined by HPLC analysis with an Aminex HPX-87P column heated to 80°C and an Altex 156 Refractive Index Detector.

7.3.6 Ethanol Production

Fermentation medium was added to the hydrolysates, sterilized at 121°C for 20 min., and inoculated with selected hexose and pentose fermenting microorganisms under microaerobic conditions at 37°C. The ethanol produced and residual sugars were determined by HPLC analysis. The fermentation medium had the following composition: $(NH_4)_2HPO_4$,0.25 g ; $MgSO_4.7H_2O$, 0.025 g ; yeast extract, 2.5 g ; and 0.1M citrate buffer, pH 4.8, was used to make the medium up to one liter (Roberto *et al.*, 1990).

The yeasts strains selected were; *Sacchromyces cerevisiae* ATCC 60868, *Pichia stipitis* NRRL Y-7124 and *Candida shehatae* NRRL Y-17024. *Candida shehatae* was chosen

because of its ability to produce ethanol at high xylose concentrations (Slininger *et al.*, 1985). *Pichia stipitis* was used because it produces high yields of ethanol from xylose without producing xylitol (Du Preez and Prior, 1985). *Sacchromyces cerevisiae* was chosen because of its high tolerance to ethanol and requires a minimum of 15% ethanol for growth. The yeast strains were obtained from the American Type Culture Collection, Rockville, Maryland, USA.

The yeast strains were maintained on YM agar at 4°C and cultivated in the following medium for inoculum production: glucose, 30 g ; yeast extract, 3 g ; malt extract, 3 g ; peptone, 5 g ; KH_2PO_4, 19 g ; $(NH_4)_2HPO_4$, 3 g ; and $MgSO_4.7H_2O$, 1.1 g. (Roberto *et al.*, 1990)

The inoculum was harvested after 36 h, centrifuged at 10 000 g and washed with 1% NaCl solution to remove residual substrate and maintain cell integrity, and then used for inoculation at the rate of 0.2% (dry weight basis)

7.4 RESULTS AND DISCUSSIONS

7.4.1 Production of a Complete Cellulase-system of *T. reseei* QMY-1

The cellulase-system of *T. reesei* has a melange of three hydrolytic enzymes that work synergistically to hydrolyze cellulose into glucose. These enzymes, traditionally referred to as endoglucanase, exoglucanase and ß-glucosidase, are termed the cellulase-system of *T. reesei.*

Cellulase-system production under SSF conditions is subject to internal environmental variations in the bioreator system since the fermentation is normally held for several weeks without pH controls. The presence of urea in the fermentation medium is not only a source of nitrogen but has effect on the pH and where the fermentation is prolonged as is invariably the case with solid-state fermentation, the initial concentration of urea in the

medium could be critical in the ability of the fermenting organism to survive and produce sufficient quantities of cellulase. In Mandels medium (Mandels and Weber, 1969), urea is used at the rate of 0.03% in liquid state fermentation systems and pH controls are easier to manipulate. Optimization of urea in solid-state fermentation is important to establish a working range of urea concentration for cellulase-system production. This is even more important when sodium hydroxide is used as the pretreatment agent since its presence also affects the pH of the fermentation substrate. The results from Table 7-1 demonstrate that in the production of optimum FPA, urea is not needed, whereas low concentration of urea (0.015%) is needed for optimum β-glucosidase production. These levels of urea concentration are lower than that used in Mandels standard medium.

Table 7-1 shows that the highest FPA was obtained when WS was pretreated with 4% NaOH (w/w) with no urea in the medium. The highest ßGA was obtained at 0.015% urea concentration and 4% NaOH. Under these conditions, over 200 filter paper units and 127 IU of ßGA were produced per g of wheat straw. Figures 7-1, 7-2 and 7-3 show the response surface curves for FPA, ßGA and their ratio. Each surface plot shows that in general cellulase activity increases with increase in NaOH concentration and decrease in urea concentration. To achieve complete cellulose hydrolysis, it is important to have high concentrations of both FPA and ßGA, and even more important is to have a FPA to ßGA ratio of about one. A ratio of ßGA to FPA close to one has been estimated to be optimum in achieving maximum cellulose hydrolysis (Stockton *et al.*, 1991, ; Breuil *et al.*, 1992).

Table 7-1: The activity of the cellulase-system of *T. reesei* QMY-1 according to second order rotatable experimental design

Run No.	Coded N (NaOH)	Coded U (Urea)	NaOH (%)	Urea (%)	FPA (IU/mL)	ßGA (IU/mL)	Ratio ßGA/FPA	FPA (IU/g)	ßGA (IU/g)
1	-1	-1	2	0.0075	2.8	0.4	0.1	69.3	11.0
2	-1	1	2	0.0225	2.9	0.4	0.1	72.7	11.0
3	1	-1	6	0.0075	6.5	6.2	1.0	161.5	156.0
4	1	1	6	0.0225	2.8	0.6	0.2	69.2	15.5
5	-1.4142	0	1.2	0.015	1.0	0.3	0.3	24.5	7.7
6	1.4142	0	6.8	0.015	4.6	2.0	0.4	115.5	50.5
7	0	-1.4142	4	0	8.2	5.1	0.6	205.7	127.0
8	0	1.4142	4	0.03	5.7	3.6	0.6	143.2	89.0
9	0	0	4	0.015	5.7	7.1	1.2	141.5	178.0
10	0	0	4	0.015	5.5	7.1	1.3	136.5	176.5
11	0	0	4	0.015	5.0	6.1	1.2	124.0	153.5
12	0	0	4	0.015	5.0	6.5	1.3	125.7	162.5
13	0	0	4	0.015	5.4	7.2	1.3	134.2	179.5

7.4.2 Hydrolysis of Delignified Wheat straw

From Table 7-1 and Figures 7-1, 7-2 and 7-3 a compromise level of NaOH and urea concentration is chosen. The enzyme harvested from these chosen levels was used for the hydrolysis of delignified wheat straw.

Some commercial enzyme were also used for the hydrolysis of DWS for comparison with the crude cellulases. The enzyme-system used from experimental run No. 3 and the commercial enzymes showed the following activities: The FPA and ßGA of experimental run No. 3 were respectively, 6.5 IU/mL and 6.2 IU/mL. The commercial enzyme, Iogen had FPA of 188.5 IU/mL and ßGA of 139.5 IU/mL; while Genencor had 194 IU/mL FPA and ßGA of 85.3 IU/mL. For hydrolysis, the quantity of these enzymes was calculated to provide 20 IU/g DWS as was the case with the crude cellulase.

The coefficients of the FPA and ßGA obtained by regression analysis are presented in Table 7-2. All but one coefficient were significant at 95% confidence level.

Table 7-2: Coefficients of FPA and βGA obtained by multiple regression analysis

Coefficient	FPA	P value	Coefficient	βGA	P value
x_0	5.294	<0.000001	x'_0	6.8	<0.00001
x_1	1.087	0.003	x'_1	1.051	0.00108
x_2	-0.886	0.00854	x'_2	-0.971	0.00155
x_{11}	-1.537	0.00063	x'_{11}	-3.018	0.00004
x_{22}	0.552	0.0738^{ns}	x'_{22}	-1.442	0.00315
x_{12}	-0.954	0.0283	x'_{12}	-1.404	0.0014

^{ns} Not significant at P < 95%. All other coefficients were significant at P < 95%

Fig. 7-1 Effect of NaOH concentration and urea on Filter Paper Activity (FPA)

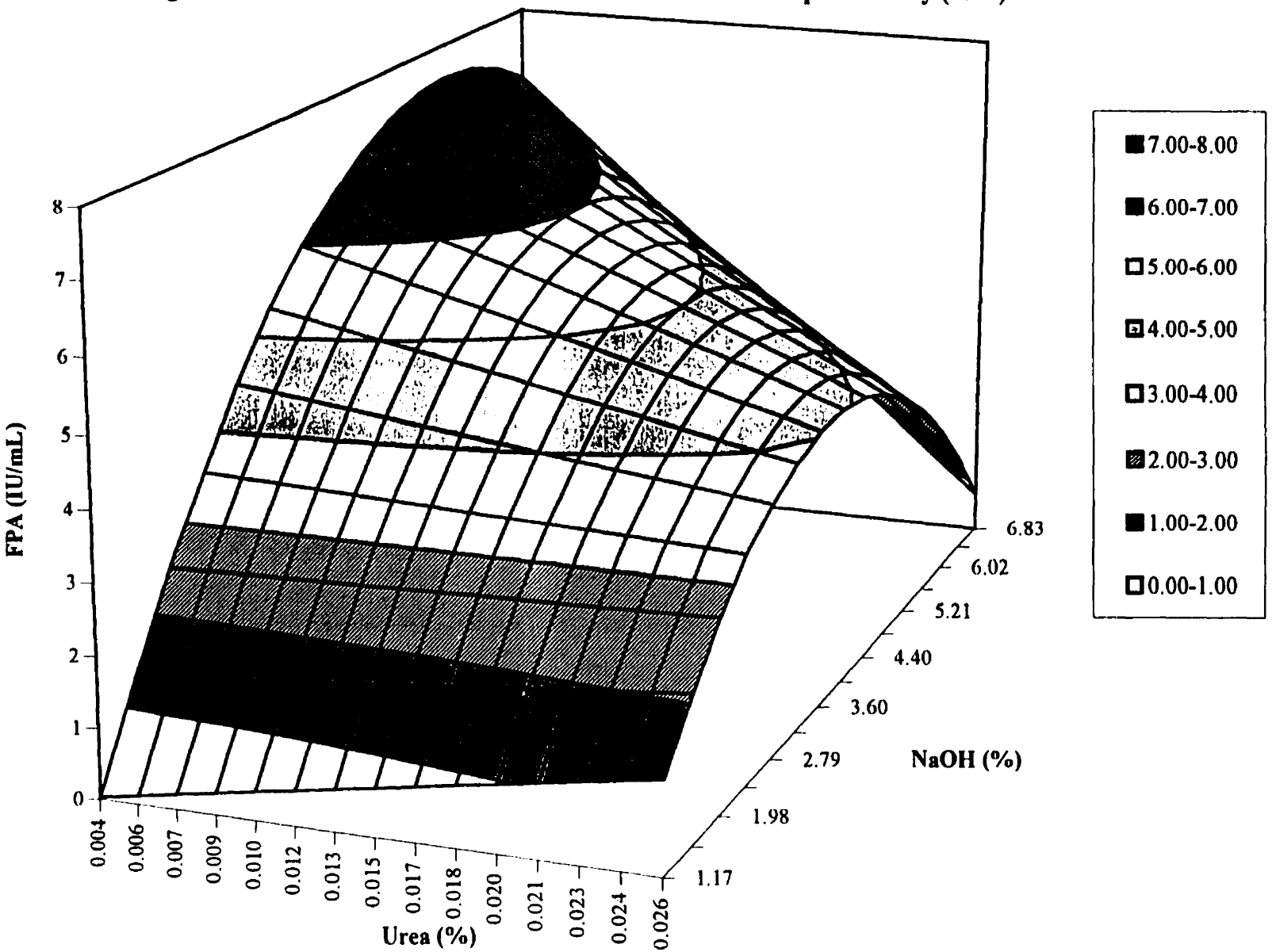


Fig. 7-2 Effect of NaOH concentration and Urea on beta-glucosidase activity

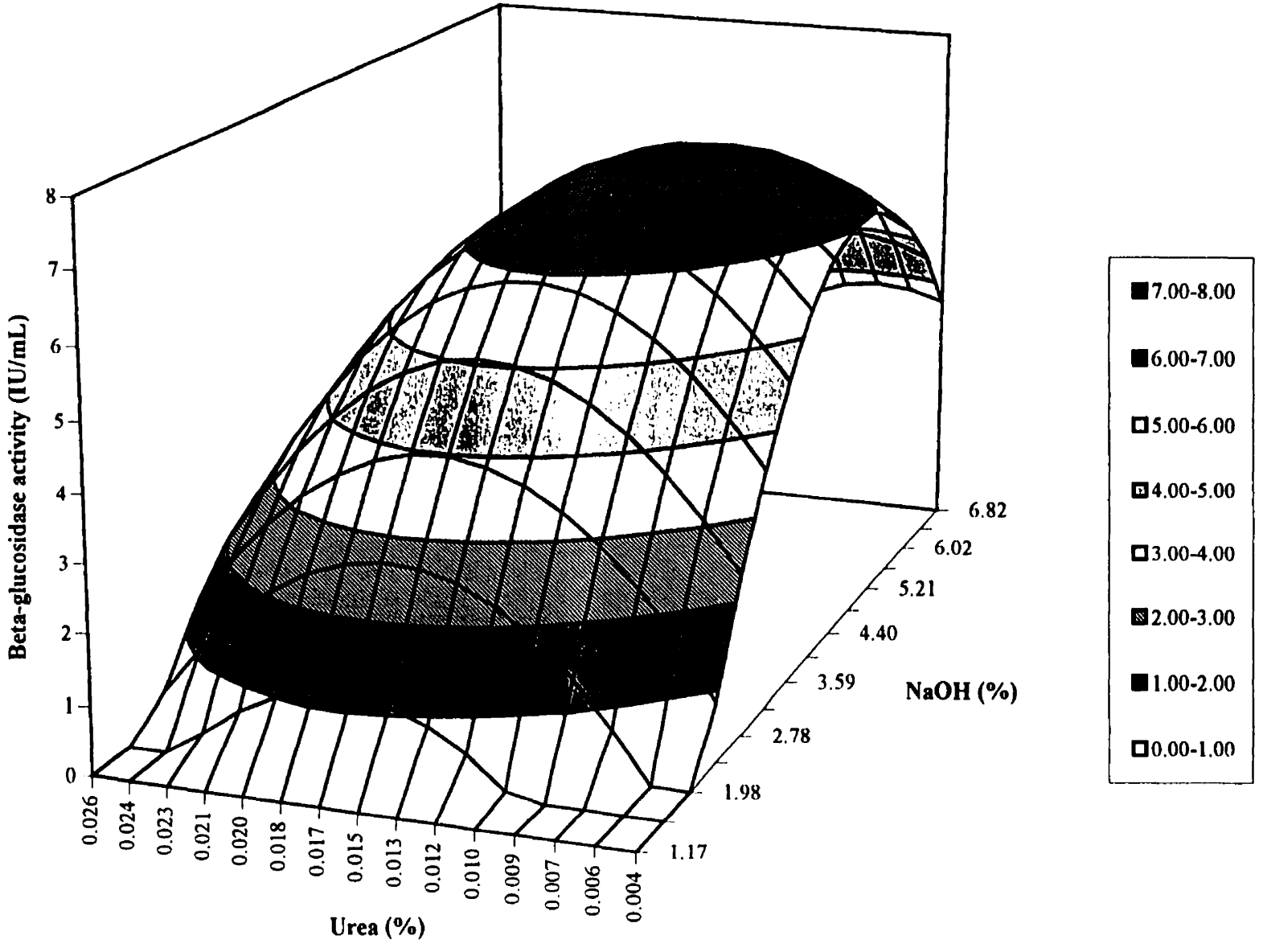


Fig. 7-3 Effect of NaOH concentration and urea on the ratio of beta-glucosidase activity to FPA

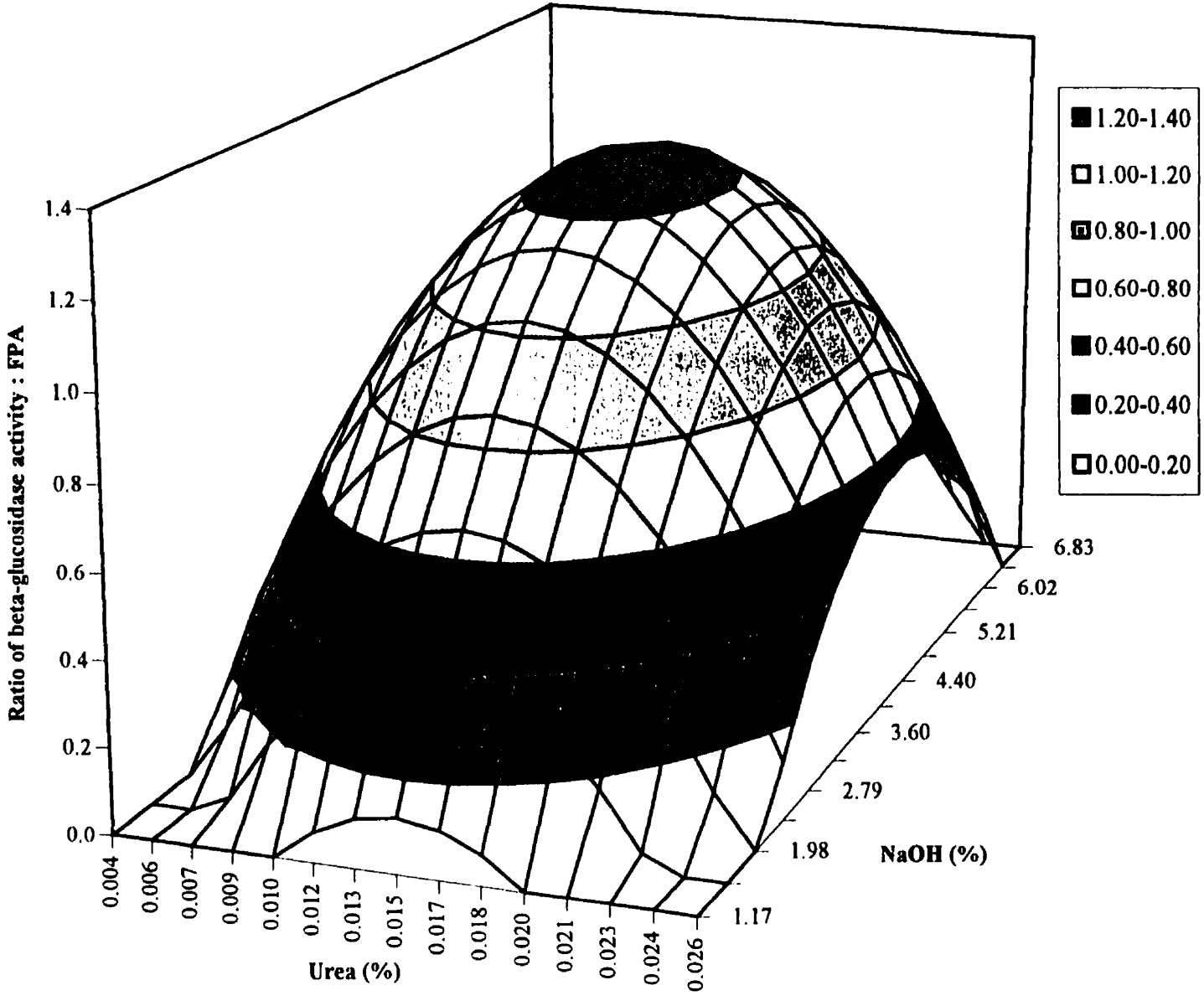


Table 7-3: Composition of hydrolysate obtained from 5% DWS after 60 h of hydrolysis

Substrate	Cellobiose g/L	Glucose g/L	Xylose g/L	Arabinose g/L	Total sugars g/L	Hydrolysis %
CEE Hydrolysate	1.61	31.34	12.51	1.65	47.11	86
CUE Hydrolysate	3.03	35.96	16.58	1.92	57.49	104[a]
Iogen Hydrolysate	0.22	38.1	8.05	1.86	48.23	88
Genencor Hydrolysate	0.32	36.67	13.77	1.2	51.96	94

[a] More than 100% hydrolysis is due to the presence of unutilized substrate of cellulose and hemicelluloses in the crude unextracted cellulase which was not accounted for in the calculation for percentage hydrolysis. Note the possible contribution of the cellulase-system to hydrolysis products as shown in Table 7-4.

The results from the hydrolysis of DWS show that all the cellulase-systems evaluated were capable of hydrolyzing over 85% of 50 g/L delignified wheat straw (Table 7-3). Maximum expected total sugars was exceeded by 5% in the fermentation system with the crude unextracted cellulase (CUE) as a consequence of additional cellulose and hemicelluloses coming along with the crude unextracted cellulase-system. It is thus evident that the unextracted cellulase-system also served as source of additional substrate for hydrolysis. More still, it may also suggest that the cellulase-system remains largely associated with the cell and functions in a cell bound manner. A direct comparison of the hydrolytic potentials of the crude extracted and unextracted cellulase-systems (Table 7-3), would further suggest that the extraction process in itself may account for the lower hydrolytic potential of the former.

It is clear that no inhibitory substances were produced during the delignification process suggesting that the method used for the delignification exposes the carbohydrate content of WS without introducing inhibitory substances. If there were any inhibitory compounds, they might have been washed out during the process of delignification. It also shows that the very high percent hydrolysis recorded indicated that β-glucosidase and exoglucanases were quick to hydrolyze fragments released by the endoglucanase since the cellobiose concentration was low. Even the apparently high cellobiose content (3g/L) from the CUE hydrolysate did not appear to have inhibitory effect on the glucanases. The very high glucose concentrations obtained also suggested lack of its feedback inhibition to the β-glucosidase.

It has been suggested that one of the constraints in the production of sugar syrups of high concentration from lignocellulosic materials is the inhibition of cellulase activity by the end-products of hydrolysis. Some researchers have reported that the inhibitory constant of (k_i) for ß-glucosidase is approximately 1 mM glucose (Ooshima *et al.*, 1985) and concentrations of glucose and cellobiose of less than 1% are estimated to strongly inhibit

the cellulase-system of *Trichoderma* (Holtzapple *et al.*, 1990; Fujii *et al.*, 1991).

The inhibitory effect of cellobiose on the cellulase-system of *Trichoderma* can, therefore, be an artifact of the quality of the cellulase-system used for the cellulose hydrolysis as the results in Table 7-3 would suggest.

Table 7-4 shows the initial composition of sugars in the cellulase-systems used for the hydrolysis of DWS and their possible contribution to the composition of the hydrolysate obtained after hydrolysis (Table 7-3).

Table 7-4: Composition of cellulase-systems used for the hydrolysis of delignified wheat straw

Cellulase-system	Composition (g/L)				
	Glucose	Cellobiose	Xylose	Total sugars	Contribution to hydrolysate[1]
Crude extracted cellulase	0.3	1.2	0.6	2.1	0.4
Commercial Iogen	33.1	194.0	-	227.1	2.3
Commercial Genencor	10.1	11.4	-	21.5	0.2

Overall, the source of the cellulase-system contributed very little to the total sugars obtained at the end of hydrolysis as shown in Table 7-4. The net change in % hydrolysis reported in Table 7-3 remains small.

[1] The amount of sugars that each cellulase-system contributed to the composition of the final hydrolysate in Table 7-3 was calculated based on the volume of each cellulase-system required to provide 20 FPA units / g DWS for hydrolysis.

Table 7-5: Residual sugars from the hydrolysate of 50 g/L DWS[1]; 30 g/L pure glucose and 30 g/L pure xylose with various yeasts.

Yeast	Substrate	Residual Sugars (g/L)					
		Cellobios	Glucose	Xylose	Arabinose	Total sugars	% Total sugars utilized
ATCC 60868	CEE - H	0.53	1.14	7.10	0.99	9.76	79
Y-7124	CEE - H	0.75	0.41	2.84	1.03	5.03	89
Y-17024	CEE - H	0.55	14.18	9.40	1.04	25.17	47
ATCC 60868 + Y-7124	CEE - H	0.90	0.46	4.95	0.70	7.01	85
ATCC 60868 + Y-17024	CEE - H	1.89	0.45	6.41	0.51	9.26	80
ATCC 60868	CUE - H	2.14	2.14	5.10	1.38	10.76	81
Y-7124	CUE - H	1.40		2.64	1.71	5.75	90
Y-17024	CUE - H	1.51	15.81	13.30	1.26	31.88	45
ATCC 60868 + Y-7124	CUE - H	1.68	0.47	8.54	0.95	11.64	80
ATCC 60868 + Y-17024	CUE - H	1.95		10.84	0.96	13.75	76
ATCC 60868	Glucose	0.45	2.42	0.61		3.48	88
Y-7124	Glucose	0.58	20.58	0.59	1.42	23.17	23
ATCC 60868 + Y-7124	Glucose	0.31	0.57	0.32		1.20	96
ATCC 60868 + Y-17024	Glucose	0.11	23.35			23.46	22
Y-7124	Xylose	0.18	0.39	14.40	0.00	14.97	50
Y-17024	Xylose	0.13	0.06	5.55	1.40	7.14	76
ATCC 60868 + Y-7124	Xylose		0.15	13.70	0.00	13.85	54
ATCC 60868 + Y-17024	Xylose			24.55		24.55	18
ATCC 60868	I - H	0.87	0.70	3.09	0.82	5.48	89
Y-7124	I - H	5.30	2.05	4.00	1.18	12.53	74
Y-17024	I - H	1.60	0.59	3.58	0.76	6.53	86
ATCC 60868 + Y-7124	I - H	3.85	7.54	3.84	1.09	16.32	66
ATCC 60868 + Y-17024	I - H	1.53	0.40	3.26	0.79	5.98	88
ATCC 60868	G - H	0.35	0.68	9.47	0.93	11.43	78
Y-7124	G - H	0.48	18.27	11.29	1.30	31.34	40
Y-17024	G - H	0.34	28.13	10.98	0.92	40.37	22
ATCC 60868 + Y-7124	G - H	0.43	0.77	9.37	0.67	11.24	78
ATCC 60868 + Y-17024	G - H	0.24	0.35	9.20	0.88	10.67	79

Legend of Table 7-5

- [1]The composition of 50 g/L DWS hydrolysates is shown in Table 7-3.
- 30 g/L each of glucose and xylose were used to reflect the minimum glucose content of the DWS hydrolysate compostion shown in Table 7-3.
- The fermenting yeasts were; *P. stipitis* (Y-7124), *S. cerevisiae* (ATCC 60868), and *Candida shehatae* (Y-17024)

CEE - H = hydrolysate from crude extracted cellulase-system of *T. reseei* QMY-1; CUE - H = hydrolysate from crude unextracted cellulase-system of *T. reesei* QMY-1; I - H = hydrolysate from Iogen commercial cellulase; G - H = hydrolysate from Genencor commercial cellulase

7.4.2 Ethanol production from the hydrolysates of DWS produced with different enzyme systems

Table 7-5 shows the details of the residual hexose and pentose components of DWS after fermentation for ethanol production with the selected yeast strains. The initial composition of the DWS hydrolysate is shown in Table 7-3.

Table 7-5 shows that over 85% of the DWS hydrolysates obtained from the cellulase-system of *T. reesei* QMY-1 were utilized by the yeasts. DWS hydrolysates were utilized to a comparable extent as pure glucose. Pure xylose was the least preferred substrate utilized.

The suitability of the hydrolysates from DWS as substrates for ethanol production was tested by fermenting them with known hexose and pentose fermenting yeast species. Table 7-6 shows that the different substrates were not fermented to the same extent by the different yeasts. The quality of the substrate also seemed important in dictating the level of ethanol fermentation. The hydrolysates obtained from the cellulase-system of *T. reesei* QMY-1 was better utilized than that from the commercial cellulases of Genencor and Iogen. *P. stipitis* Y-7124 was the best at converting the DWS hydrolysate into ethanol. Pure glucose and xylose were less preferred than the DWS hydrolysates by all the yeast strains for ethanol production suggesting that these yeast species especially *P. stipitis* Y-7124 and *S cerevisiae* ATCC 60868 can efficiently ferment hexose and pentose mixtures.

S. cerevisiae and *P. stipitis* were better at converting the glucose and xylose components of the DWS than *C. shehatae*. Co-cultures of *S. cerevisiae* and *P. stipitis* did not improve ethanol production beyond their individual capabilities of fermentation. Glucose was utilized most suggesting that it is the preferred substrate by the selected yeast strains. *P. stipitis* proved to be the most suitable yeast for ethanol production from the mixed hexoses and pentoses obtained from DWS.

Of all the cellulase-systems examined, crude unextracted cellulase-system produced the highest percent of hydrolysis of the delignified wheat straw that subsequently produced the highest ethanol (0.51 g ethanol/g total sugars) with *P. stipitis* Y-7124 having a volumetric productivity of 0.42 g/L/h. These yields were obtained from about 90% utilization of the DWS. This translates to 22.5 g/L of ethanol from 50 g/L DWS.

When all the cellulase-systems were evaluated for their abilities to ferment glucose, the crude unextracted cellulase-system was again the best with over 90% of the theoretical yield. The yields obtained with the crude extracted cellulase-system were comparable to those of commercial cellulases from Iogen and Genencor.

7.5 CONCLUSION

Wheat straw pretreated with mild sodium hydroxide was suitable for the production of high quality cellulase-system from *T. reesei* QMY-1 for the efficient hydrolysis of delignified wheat straw.

Crude unextracted cellulase-system appears to be the ultimate answer to efficient cellulose hydrolysis and ethanol production with practical commercial application since it saves cost in enzyme production by eliminating the extraction process. The hydrolysates produced from the cellulase-system of *T. reesei* QMY-1 were better utilized as fermentation substrates for ethanol production than those from some commercial cellulases.

Table 7-6: Comparison of ethanol yields of 50 g/L DWS hydrolysates; 30 g/L glucose and 30 g/L xylose by *S.cerevisiae* (ATCC 60868) *P. stipitis* (Y-7124) and *C. shehatae* (Y-17024) under microaerobic conditions for 60 h.

Yeast	Substrate	Ethanol g/L	Ethanol yield g /g DWS	% conversion g /g DWS
ATCC 60868	CEE hydrolysate	15.72	0.44	87
Y-7124	CEE hydrolysate	15.36	0.38	74
Y-17024	CEE hydrolysate	2.68	0.13	26
60868 + Y-7124	CEE hydrolysate	16.61	0.43	85
60868 + Y-17024	CEE hydrolysate	12.92	0.35	68
ATCC 60868	CUE hydrolysate	12.26	0.43	85
Y-7124	CUE hydrolysate	25.22	0.51	99
Y-17024	CUE hydrolysate	5.88	0.25	49
60868 + Y-7124	CUE hydrolysate	19.79	0.45	89
60868 + Y-17024	CUE hydrolysate	15.51	0.37	73
ATCC 60868	Glucose	11.15	0.41	81
Y-7124	Glucose	4.28	0.48	95
Y-17024	Glucose	12.14	0.42	82
60868 + Y-7124	Glucose	0.61	0.09	18
ATCC 60868	Xylose	5.35	0.35	69
Y-7124	Xylose	6.28	0.26	50
Y-17024	Xylose	1.77	0.11	21
60868 + Y-7124	Xylose		0.00	0
ATCC 60868	logen Hydrolysate	15.05	0.35	69
Y-7124	logen Hydrolysate	12.13	0.30	59
Y-17024	logen Hydrolysate	13.43	0.32	62
60868 + Y-7124	logen Hydrolysate	11.06	0.31	61
60868 + Y-17024	logen Hydrolysate	12.31	0.29	56
ATCC 60868	Genencor Hydrolysate	12.23	0.30	59
Y-7124	Genencor Hydrolysate	5.25	0.25	49
Y-17024	Genencor Hydrolysate	0.21	0.02	4
60868 + Y-7124	Genencor Hydrolysate	12.41	0.31	60
60868 + Y-17024	Genencor Hydrolysate	14.10	0.34	68

The percent conversion of DWS was determined by dividing the ethanol produced by the DWS concentration and multipling by 196. This calculation accounts for the stoichiometry of ethanol production and assumes 90% conversion of sugars into ethanol.

CHAPTER 8

THE OPTIMIZATION OF ETHANOL PRODUCTION BY *SACCHROMYCES CEREVISIAE* (ATCC 60868) AND *PICHIA STIPITIS* Y-7124: A RESPONSE SURFACE MODEL OF WHEAT STRAW CONCENTRATION AND CELLULASE-SYSTEM LOADING FOR SIMULTANEOUS HYDROLYSIS AND FERMENTATION.

8.0 CONNECTING STATEMENT

Chapter 8 looks at the other method for ethanol production, simultaneous hydrolysis and fermentation, unlike the separate hydrolysis and fermentation method that was employed in chapter 7. The volumetric productivities (g ethanol/L/h) from both processes could be a deciding factor in the choice of one process over the other for ethanol production from lignocelluloses. Advantage was taken of this latter approach to optimize the substrate and cellulase loading so as to show optimum ethanol production with different yeast species. Results obtained from Chapters 7 and 8 are the ultimate indicators of the efficiencies of processes aimed at obtaining quality cellulase-systems described in the previous chapters.

Note : This chapter constitutes the text of a manuscript submitted for publication as follows : Awafo, V.A., Chahal, D.S. and Simpson, B.K. 1997. The optimization of ethanol production by *Sacchromyces cerevisiae* (ATCC 60868) and *Pichia stipitis* Y-7124: A response surface model of wheat straw concentration and cellulase-system loading for simultaneous hydrolysis and fermentation. Appl. Biochem. Biotechnol.

Contribution of co-authors : Chahal, D.S. and Simpson, B.K. (Thesis co-supervisors).

8.1 ABSTRACT

Optimization models are useful in ascertaining optimum parameters as they relate to a particular response. Ethanol production with different cellulase-systems and substrate concentrations were subjected to this model and the results have revealed that crude unextracted cellulase-systems are more efficient than crude extracted cellulase-systems in producing the hydrolysates for ethanol production with *Pichia stipitis* (Y-7124), *Sacchromyces cerevisiae* (AT 60868) and their mixture. Response surface plots showed that 80-90% of high concentrations of delignified wheat straw up to 11% could be converted into ethanol by simultaneous hydrolysis with enzyme loading of 16 IU/mL and fermentation with *P. stipitis*. *P. stipitis* was found to be the most suitable yeast for the fermentation of mixed hexose and pentose sugars in the wheat straw hydrolysate.

8.2 INTRODUCTION

Sacchromyces cerevisiae ATCC 60868 and *Pichia stipitis* Y-7124 are yeast that are capable of fermenting hexose and pentose sugars, respectively, into ethanol. The former has high tolerance to ethanol concentration and the latter to high xylose concentration (Du Preez and Prior, 1985).

A mixture of hexoses and pentoses and other compounds, as occurs, in lignocellulosic hydrolysates is not easily fermented by most yeast species. In the literature, three types of studies with lignocellulosic materials as potential substrates for ethanol production are available. In one category, lignocelluloses are studied on the sole basis of their potential for either enzymatic or chemical hydrolysis into monomeric sugars notably, hexoses and pentoses. In most of these studies involving enzymatic hydrolysis, the general preference

has been to pretreat the substrate, chemically or physically, and subject it to hydrolysis with commercial cellulases, meicelase, celluclast, novozym etc (Nidetzky *et al.*, 1993; Esterbauer *et al.*, 1991; Moniruzzaman, 1996). The suitability of these hydrolysates as fermentation substrates in some of these studies is not reported and yet it has been abundantly demonstrated that pretreatments produce inhibitory compounds in the hydrolysates (Van Zyl *et al.*, 1991). In the second category of study which attempts to tackle the first problem, the former category of study is carefully repeated and the hydrolysates is then subjected to fermentation for ethanol production with suitable yeast (Delgenes *et al.*, 1990; Lindén *et al.*, 1992). These studies also suffer from the point of view of practical applications since commercial cellulases are cost prohibitive at least for now (Perez *et al.*, 1980). The last category of study which should normally address itself to these problems involves enzymatic hydrolysis of pretreated lignocelluloses with cellulase-systems obtained from pretreated lignocelluloses and fermentation of their hydrolysates with suitable yeast strains for ethanol production.

The general survey of the literature reveals scanty information in this direction. This has generally been so because liquid state fermentation which has been at the core of cellulase production for some time now produces cellulase-systems largely deficient in the ß-glucosidase. Liquid state fermentation also has some limitations in utilizing large quantities of lignocelluloses even under fed-batch fermentation conditions. Solid-state fermentation largely overcomes limitations of ß-glucosidase since large quantities of lignocelluloses per unit volume can be used and the fermentation can be performed at relatively little cost. More still, unutilized substrate at the end of every solid-state fermentation process is also a potential substrate for subsequent hydrolysis into glucose. High filter paper activity and ß-glucosidase activity with the ratio of the latter to the former close to one is now considered to be important in alleviating feedback inhibition

of cellobiose. In some parallel studies, yeast species are evaluated for their performance on pure substrates such as glucose and xylose (Delgenes *et al.*, 1991). These results cannot, however, be extrapolated onto lignocellulosic substrates because of their complex nature as aforementioned.

This study reports the optimization of cellulase loading and delignified wheat straw concentration in ethanol production with *S. cerevisiae* and *P. stipitis*. Central composite rotatable design (Schmidt and Launsby, 1992) was chosen to optimize the cellulase loading and substrate concentration because it is more useful in practice than other designs and requires less number of experimental points to determine polynomial coefficients and also measures the lack of fit of the resulting equation. It is necessary to optimize this system because the literature shows a wide range of cellulase activities that are normally used ranging from 5 to 100 FPU/g but more often 20 FPU/g (Nidetzky *et al.*, 1993; Esterbauer *et al.*, 1991; Wright, 1988).

Optimization of experimental parameters is an important step in evaluating an efficient process for fuel ethanol production. This has become even more important in the light of the fact that the literature contains several reports of fuel ethanol production with several microorganisms utilizing different lignocelluloses at various concentration levels. Furthermore, as more and more realistic substrates are being examined for fuel ethanol production, optimization of individual processes will provide information for direct comparison with others to ascertain scale -up capabilities.

8.3 MATERIALS AND METHODS

8.3.1 Cellulase-system production

Wheat straw was pretreated with 4% NaOH and sterilized at 121 °C for one hour. *T. reesei* QMY-1 was grown on this substrate with Mandels medium (Mandels and Weber, 1969) under solid-state fermentation conditions for 21 days. The harvested cellulase-system was extracted in citrate buffer (pH, 4.8) and the crude cellulase-system was then used for the hydrolysis of delignified wheat straw. The filter paper activity (FPA) and ß-glucosidase activity (ßGA) of the crude cellulase-system were determined by the method approved by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987).

8.3.2 Fermenting microorganisms

Sacchromyces cerevisiae ATCC 60868 and *Pichia stipitis* Y-7124 were obtained from the American Type Culture Collection, Rockville, Maryland, USA and maintained on YM agar at 4°C. They were cultivated in the following medium for inoculum production: glucose, 30 g/L, yeast extract, 3 g/L, malt extract, 3 g/L, peptone, 5 g/L, KH_2PO_4, 19 g/L, $(NH_4)_2HPO_4$,, 3 g/L, $MgSO_4.7H_2O$, 1.1 g/L.

8.3.3 Simultaneous Hydrolysis and Fermentation (SHF)

Wheat straw was delignified according to the method of Toyama and Ogawa (1972) and

used as the substrate for ethanol production. Delignified wheat straw (DWS) containing about 90% holocellulose was subjected to simultaneous hydrolysis and fermentation (SHF) using a second order central composite design to study the effect of DWS concentration (5-10% dry weight basis) and cellulase-system loading (20-40 IU/g) on ethanol production with *S. cerevisiae*, ATCC 60868 and *P. stipitis* Y-7124. Five replicates at the central points were performed to determine standard error and there were four kernel points and four star points with $a = \sqrt{2}$. All results shown were mean values of duplicate determinations.

The independent variables were coded as

$W = (w - 7.5)/2.5$

$C = (c - 30)/10$

Where W and C are the respective normalized values of delignified wheat straw and cellulase concentrations

w = Delignified wheat straw (DWS) concentration (%)

c = Cellulase-system loading (IU/g DWS)

The constants, 7.5 and 30 are the respective averages for the actual values of the sum of the high and low concentrations of DWS and cellulase-system loading

while, the values 2.5 and 10 represent the respective averages for the actual values of the differences between the high and low concentrations of DWS and cellulase-system loading.

The general equation of the model takes the form:

$$E = X_0 + X_1(W) + X_2(C) + X_{11}(W^2) + X_{22}(C^2) + X_{12}(W)(C) \qquad (1)$$

where E = Ethanol yield (g/L).

The experimental design was as shown in Table 8-1. This model was used for both crude extracted cellulase-system (CEC) and crude unextracted cellulase-system (CUC). CEC is the supernatant obtained after centrifugation (x 11 000 g) of the solid fermented cellulase.

The fermentation medium (Roberto *et al.*, 1990) was mixed with the DWS and equivalent amounts of the CEC were added based on the filter paper activity per gram of the DWS according to the model in Table 8-1. CUC was also supplied on the basis of the FPA of the extracted enzyme per gram fermented substrate. The fermenting yeast, *S. cerevisiae*, and *P. stipitis* were harvested from their respective exponential growth phases and added to each experimental setup at the concentration level of 2% (dwb/v). Each experimental setup was held in a water bath at 37°C for 72 h. Cellulase-systems are known to be most active between 40 °C- 45°C (Duff and Murray, 1996) while the yeast are most active below 40°C (Olsson and Hahn-Hägerdal, 1993; Ballesteros *et al.*, 1993). The compromise temperature for the simultaneous hydrolysis and fermentation was pegged at the latter temperature and fermentation prolonged to 72 h to give more time for the cellulase-system to hydrolyze more DWS and minimize possible contamination from the cellulase-system.

After 72 h of simultaneous hydrolysis and fermentation, the hydrolysate was centrifuged for 30 min at 11 000 g and subjected to HPLC analysis with an Aminex HPX-87P column heated to 80°C and an Altex 156 Refractive Index Detector. Residual sugars and ethanol yields were expressed as g/L.

Table 8-1. Orthogonal design of total available sugars in DWS and cellulase-system loading.

Run No.	DWS Normalized (W)	Cellulase-system Normalized (C)	DWS Actual value (w)	DWS Initial Total Sugars (g/L)	Cellulase-system Actual value (c) (IU/g)
1	-1	-1	5	47	20
2	1	-1	10	94	20
3	-1	1	5	47	40
4	1	1	10	94	40
5	-1.414	-1.414	4	38	30
6	1.414	1.414	11	104	30
7	0	0	7.5	71	16
8	0	0	7.5	71	44
9 - 13	0	0	7.5	71	30

* = [glucose + xylose + cellobiose + arabinose]

8.4 RESULTS AND DISCUSSION

8.4.1 Yield parameters after simultaneous hydrolysis and fermentation (SHF) of DWS with crude extracted and crude unextracted cellulase-systems of *T. reesei* QMY-1 for fuel ethanol production

The composition of the cellulase-system obtained under solid-state fermentation conditions that was used for the SHF was as follows: FPA, 6.5 IU/mL; ßGA, 6.2 IU/mL; and the ratio of the latter to former was 1.0. A good ratio of about one is important in ensuring that when the endo- and exo- components of the cellulase-system catalyze cellulose hydrolysis into cellobiose there is complete hydrolysis of cellobiose to glucose. This is kinetically important in two ways; it alleviates end-product inhibition of cellobiose since concentrations of as low as 1% are inhibitory (Holtzapple *et al.*, 1990; Fujii *et al.*,1991). Secondly, by ensuring optimum cellobiose hydrolysis into glucose, more sugars diffuse to the cells of the yeasts for ethanol synthesis.

Tables 8-2 to 8-4 show ethanol yields obtained when crude extracted cellulase (CEC) was used to catalyze DWS hydrolysis and fermented with *S. cerevisiae* and *P. stipitis* in SHF. The percent actual yields reported in the Tables were obtained by dividing the g/L ethanol by the DWS concentration in g/L supplied and multiplying by 196 to account for the stoichiometry of ethanol production. Tables 8-5 to 8-7 on the other hand show similar yield parameters obtained with crude unextracted cellulase (CUC).

Fig. 8-1 shows chromatograms obtained from HPLC analysis after using both CEC and CUC for the hydrolysis of 5% DWS. CUC produced higher proportions of all the

reducing sugars that were analyzed than those obtained with the CEC suggesting a better performance by the CUC. Part of the increase could also have resulted from the presence of additional cellulose and hemicelluloses in the CUC that were not fermented.

8.4.2 Optimization of ethanol yields from the second order experimental design

Tables 8-2, 8-3 and 8-4 show the respective yield parameters for ethanol production with *S. cerevisiae*, *P. stipitis* and the combination of *S. cerevisiae* and *P. stipitis* under simultaneous hydrolysis and fermentation conditions with delignified wheat straw (DWS) and crude extracted cellulase-system from *Trichoderma reesei*. Tables 8-2, 8-3 and 8-4 also show the residual sugars obtained after the simultaneous hydrolysis and fermentation.

In the optimization of DWS and cellulase loading with *S. cerevisiae*, the optimum ethanol concentration of ethanol (23.9 g/L) and equivalent yield of 0.33 g ethanol/g DWS was attained at a substrate concentration of 10% DWS and CEC loading of 20 IU/g as shown in the orthogonal design in Table 8-1. Overall, *S. cerevisiae* was capable of yielding about 68% ethanol from 10 % DWS.

On the other hand, with *P. stipitis*, several combinations of DWS concentration and cellulase loadings produced over 80% ethanol. The highest ethanol yields (90 and 91%) were obtained with 5% DWS, 40 IU/g CEC and 4% DWS, 30 IU/g CEC respectively. *P. stipitis* produced higher ethanol yields (0.43 g ethanol/g DWS) compared to 0.33 g ethanol/g DWS from *S. cerevisiae* (Tables 8-2 and 8-3).

When DWS was fermented with a mixture of *S. cerevisiae* and *F. stipitis*, higher ethanol yields were obtained compared to *S. cerevisiae* and *P. stipitis* alone at certain substrate and cellulase loading levels. The highest ethanol yield of 0.45 g/g DWS was obtained with the combinations of 5% DWS and 20 IU/g CEC and 7.5% DWS with 16 IU/g CEC loading (Table 8-2)

Comparison of similar results obtained with CUC loadings as shown in Tables 8-5, 8-6 and 8-7 show that ethanol yields were slightly higher with both yeast and their combination. Residual sugars obtained from the SHF with CEC were higher than those correspondingly obtained with CUC. These results would seem to suggest that CUC produced a higher turn over rate of reducing sugars which were subsequently converted to ethanol by the yeast. It could also mean that CEC produced lower quantities of reducing sugars because of its quality compared to that of CUC. The hypotheses may, therefore, be that cellulases desorption, as expected in the extraction process, does not match its resorption process during cellulose hydrolysis and hence the lowering of its reactivity. The close contact of CUC and cellulose during hydrolysis could enhance its desorption, resorption and reactivity.

Fig. 8-2 shows ethanol yields from the fermenting yeast under different substrate and cellulase loadings. At all levels of substrate and cellulase loadings, *P. stipitis* appeared to produce the highest ethanol yields except at 7.5% DWS when a mixture of *P. stipitis* and *S. cerevisiae* performed better. At 10% DWS, doubling the cellulase loading from 20 IU/g to 40 IU/g did not increase the ethanol yields with either *S. cerevesiae*, *P. stipitis* and their combination. This was similarly reflected at 5% DWS concentration.

Fig. 8-3 shows the comparison of residual sugars to total sugars available for conversion into ethanol with CEC. At all levels of substrate and cellulase loadings, *S. cerevesiae*, *P.*

stipitis and their combination were capable of utilizing DWS for ethanol production to different extents. Overall, combination of *S.cerevisiae* and *P. stipitis* appeared to have produced less residual sugars compared to either *S. cerevisiae* and *P. stipitis* alone at high DWS concentrations of 10 and 11%. At lower DWS, there was no apparent difference in the residual sugars from all both *P. stipitis* and *S. cerevisiae*.

Surface plots of actual ethanol yields for CEC and CUC are shown in Figs. 8-4, 8-5, and 8-6. Figs. 8-4 and 8-7 show that *S. cerevisiae* was not capable of producing over 80% ethanol from DWS with both CEC and CUC. Both figures show that high ethanol yields are obtained by increasing both the cellulase loadings and substrate concentrations. Figs. 8-5 and 8-8 show the respective trends with CEC and CUC and *P. stipitis*. Both show that 90-100% ethanol production could be achieved. This is contrast to *S. cerevisiae* which could only give maximum ethanol yields of 70-80%. Fig. 8-5 shows that 90-100 % ethanol could be achieved with CEC loading of 20 IU/g and 4-4.5% DWS. On the other hand, Fig. 8-8 shows that a minimum of CUC loading of 24 IU/g is needed to obtain between 90-100% hydrolysis. However, similar results are obtained with CUC loading of 30 IU/g and 7% DWS concentration. Finally, a mixture of *S.cerevisiae* and *P. stipitis* was also capable of attaining between 90 and 100% ethanol but required low levels of both CEC loading (16-18 IU/g) and DWS concentration (4-4.5%) (Fig. 8-6) as compared to CUC loadings (16-32 IU/g) and broader DWS concentrations (4-5-5%) as illustrated in Fig 8-9.

8.5 CONCLUSION

The optimization of ethanol production from both crude extracted cellulase-systems and crude unextracted cellulase-systems has shown that both enzyme-systems are capable of

yielding over 90% ethanol with *P. stipitis*. The crude unextracted cellulase-system appeared to tolerate higher substrate concentrations than the crude extracted cellulase-system. The optimization model also showed that *P. stipitis* was better at producing ethanol than *S. cerevisiae* with delignified wheat straw as substrate.

Table 8-2. Optimization of DWS and CEC for ethanol production under simultaneous hydrolysis and fermentation: Yield parameters with *Sacchromyces cerevisiae* (ATCC 60868)

	Residual Sugars (g/L)							
Run No.	Cellobiose	Glucose	Xylose	Arabinose	Total sugars	Ethanol (g/L)	Yield (g/g)	Yield (%)
1	1.3	2.9	1.7	0.47	6.37	10.27	0.25	51
2	1.12	1.21	17.7	1.6	21.63	23.9	0.33	68
3	0.72	3.12	1.03	1.06	5.93	12.05	0.29	59
4	1.07	3.57	12.99	1.6	19.23	17.3	0.23	48
5	0	0.27	2.98	0	3.25	8.99	0.26	55
6	0	8.81	13.28	1.65	23.74	22.39	0.28	59
7	0	8.93	5.22	1.23	15.38	17.1	0.31	65
8	0.61	1.07	3.72	1.63	7.03	18.5	0.29	59
9	0.83	1.76	7.52	1.1	11.21	17.38	0.29	60
10	1.65	0	7.83	0.97	10.45	18.29	0.3	62
11	2.24	0.52	7.28	1.23	11.27	18.47	0.31	62

The ethanol yield was determined by dividing the g/L ethanol by substrate concentration utilized. The percent conversion was determined by dividing the g/L ethanol by the substrate concentration (glucose + xylose) utilized and multiplying by 196 to account for the stoichiometry of ethanol production and assumes 90% conversion of sugars to ethanol by yeast leaving 10% for cell growth.

Table 8-3. Optimization of DWS and CEC for ethanol production under simultaneous hydrolysis and fermentation: Yield parameters with *Pichia stipitis* (Y-7124)

Run No.	Residual Sugars (g/L)					Ethanol (g/L)	Yield (g/g)	Yield (%)
	Cellobiose	Glucose	Xylose	Arabinose	Total sugars			
1	1.36	0.42	6.12	1.03	8.93	16.25	0.43	85
2	1.08	2.27	14.96	1.82	20.13	25.03	0.34	70
3	0.26	0.77	4.15	0.58	5.76	17.95	0.43	90
4	1.12	2.43	19.72	1.53	24.8	22.5	0.32	67
5	0.52	0.2	6.4	0	7.12	13.22	0.43	91
6	0.5	3.12	18.96	1.53	24.11	28.45	0.36	75
7	1.27	1.85	9.22	1.07	13.41	16.85	0.29	60
8	0.27	2.06	4.58	1.2	8.11	21.22	0.34	70
9	0	4.47	15.03	1.51	21.01	18.52	0.37	78
10	0.41	5.41	17.82	1.14	24.78	17.06	0.37	79

Yield was calculated as in Table 8-2

Table 8-4. Optimization of DWS and CEC for ethanol production under simultaneous hydrolysis and fermentation: Yield parameters with mixture of *Sacchromyces cerevisiae* (ATCC 60868) and *Pichia stipitis* (Y-7124)

Run No.	Residual Sugars (g/L)					Ethanol (g/L)	Yield (g/g)	Yield (%)
	Cellobiose	Glucose	Xylose	Arabinose	Total sugars			
1	0.2	0	15.1	1.28	16.58	13.74	0.45	94
2	5.99	1.02	11.28	1.5	19.79	25.64	0.34	67
3	0.43	0	5.43	0.64	6.5	10.11	0.25	52
4	0	1.27	14.04	1.25	16.56	17.16	0.22	46
5	0.31	0.48	10.61	0.74	12.14	10.57	0.41	86
6	0	1.1	11.94	1.05	14.09	19.79	0.22	46
7	0.87	1.29	9.53	1.01	12.7	26.12	0.45	93
8	0.38	4.16	6.27	1.16	11.97	18.36	0.31	65
9	0	6.25	11.18	0.95	18.38	16.42	0.31	67
10	1.21	0.73	9.44	0.83	12.21	15.65	0.27	55

Yield was calculated as in Table 8-2

Table 8-5. Optimization of DWS and CUC for ethanol production under simultaneous hydrolysis and fermentation: Yield parameters with *Sacchromyces cerevisiae* (ATCC 60868)

	Residual Sugars (g/L)							
Run No.	Cellobiose	Glucose	Xylose	Arabinose	Total sugars	Ethanol (g/L)	Yield (g/g)	Yield (%)
1	0.57	1.39	1.55	0.72	4.23	12.74	0.3	61
2	1.1	0.81	8.8	0.77	11.48	27.97	0.34	70
3	0.75	1.24	1.17	1.61	4.77	12.67	0.3	60
4	1.25	2.97	14.11	2.01	20.34	19.51	0.26	54
5	0.6	0.17	2.19	0.23	3.19	10.56	0.31	63
6	0.52	5.22	13.86	2.25	21.85	23.51	0.29	60
7	1.31	0.55	5.49	0.67	8.02	16.27	0.26	53
8	0	0	6.65	1.46	8.11	19.89	0.32	66
9	0.23	1.97	9.97	1.03	13.2	19.17	0.33	70
10	0.2	3.18	11	0.98	15.36	18.29	0.33	69
11	0.14	2.4	10.64	1.3	14.48	18.97	0.34	71

Yield was calculated as in Table 8-2

Table 8-6. Optimization of DWS and CUC for ethanol production under simultaneous hydrolysis and fermentation: Yield parameters with *Pichia stipitis* (Y-7124)

	Residual Sugars (g/L)							
Run No.	Cellobiose	Glucose	Xylose	Arabinose	Total sugars	Ethanol (g/L)	Yield (g/g)	Yield (%)
1	1.51	0.38	5.56	1.37	8.82	16.85	0.44	87
2	1.97	0.49	6.15	2.21	10.82	26.18	0.31	63
3	1.02	0.22	4.73	2.34	8.31	18.95	0.49	95
4	1.76	0.38	6.87	2.3	11.31	27.67	0.33	67
5	0	0.16	7.09	0.59	7.84	12.98	0.43	91
6	1.13	1.51	12.16	3.06	17.86	29.78	0.35	70
7	1.31	1.78	10.22	2.15	15.46	18.56	0.34	68
8	1.16	1.95	7.51	1.32	11.94	23.47	0.4	82
9	0.47	3.21	16.45	1.6	21.73	20.84	0.43	89
10	0.35	2.97	17	1.47	21.79	21.06	0.43	90
11	0.41	3.17	15.79	1.16	20.53	21.06	0.42	88

Yield was calculated as in Table 8-2

Table 8-7. Optimization of DWS and CUC for ethanol production under simultaneous hydrolysis and fermentation: Yield parameters with *Pichia stipitis* (Y-7124) and *Sacchromyces cerevisiae* (ATCC 60868) mixture.

Run No.	Residual Sugars (g/L)					Ethanol (g/L)	Yield (g/g)	Yield (%)
	Cellobiose	Glucose	Xylose	Arabinose	Total sugars			
1	1.1	2.3	11.21	2.11	16.72	13.72	0.45	89
2	2.33	1.21	13.28	1.71	18.53	26.57	0.35	71
3	1.14	0.65	8.05	1.04	10.88	13.29	0.37	74
4	1.53	0.78	16.48	1.27	20.06	20.16	0.27	56
5	0.57	0.35	9.58	0.92	11.42	11.75	0.45	92
6	2.14	0.98	12.04	1.21	16.37	20.59	0.24	48
7	1.17	1.08	12.53	0.89	15.67	25.14	0.46	94
8	1.38	3.55	7.27	2.16	14.36	20.63	0.37	74
9	0.84	6.87	10.79	0.78	19.28	18.77	0.37	76
10	1.12	6.73	9.44	0.77	18.06	19	0.36	75
11	0.92	5.87	10.11	0.84	17.74	18.91	0.36	74

Yield was calculated as in Table 8-2

Fig. 8-1 Typical HPLC chromatograms of reducing sugars obtained from crude extracted (CEC) and crude unextracted cellulase-systems (CUC) of *T.reesei* QMY-1 on hydrolysis of 5% DWS

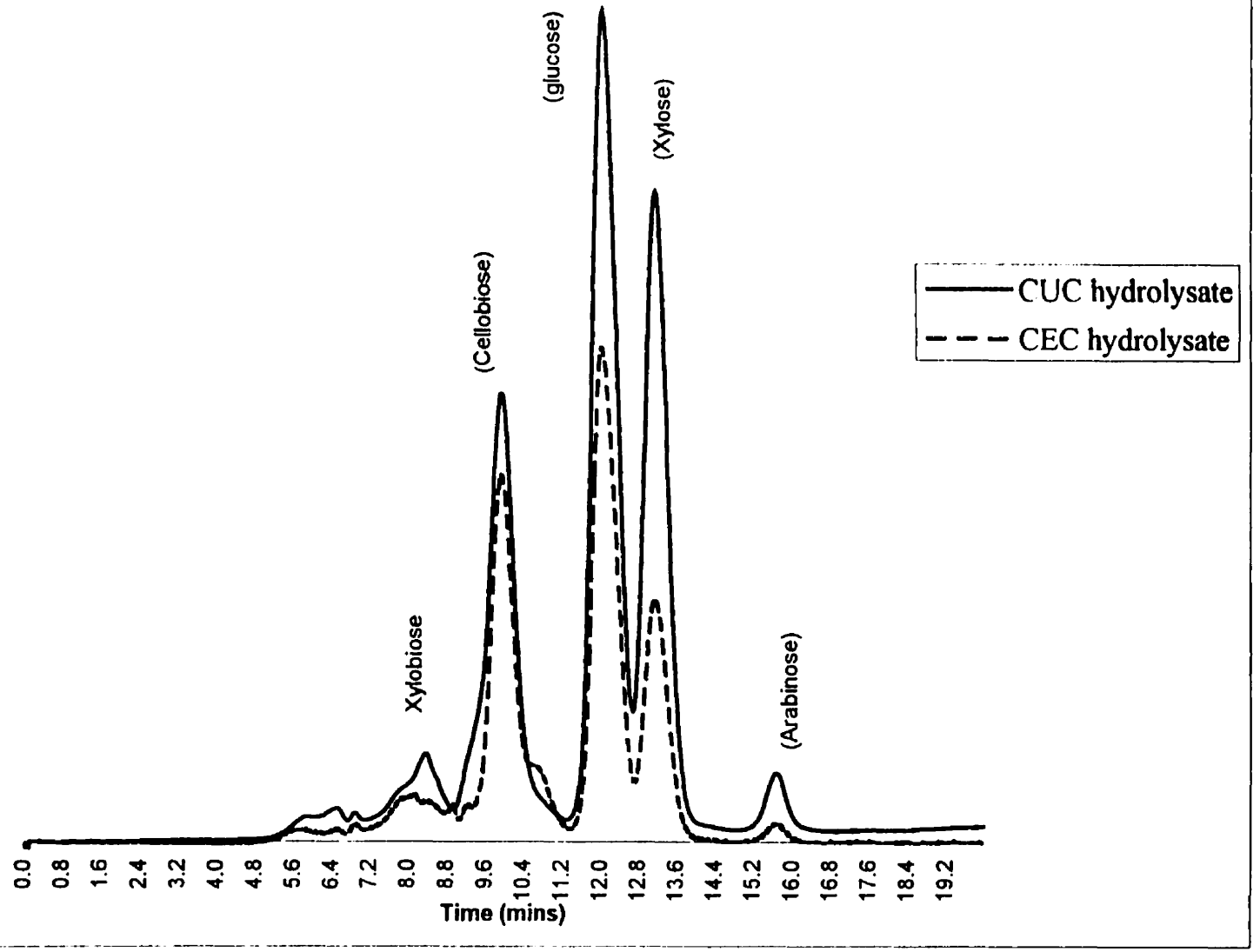


Fig 8-2 Effect of different fermenting yeast on ethanol production with different substrate and cellulase concentrations

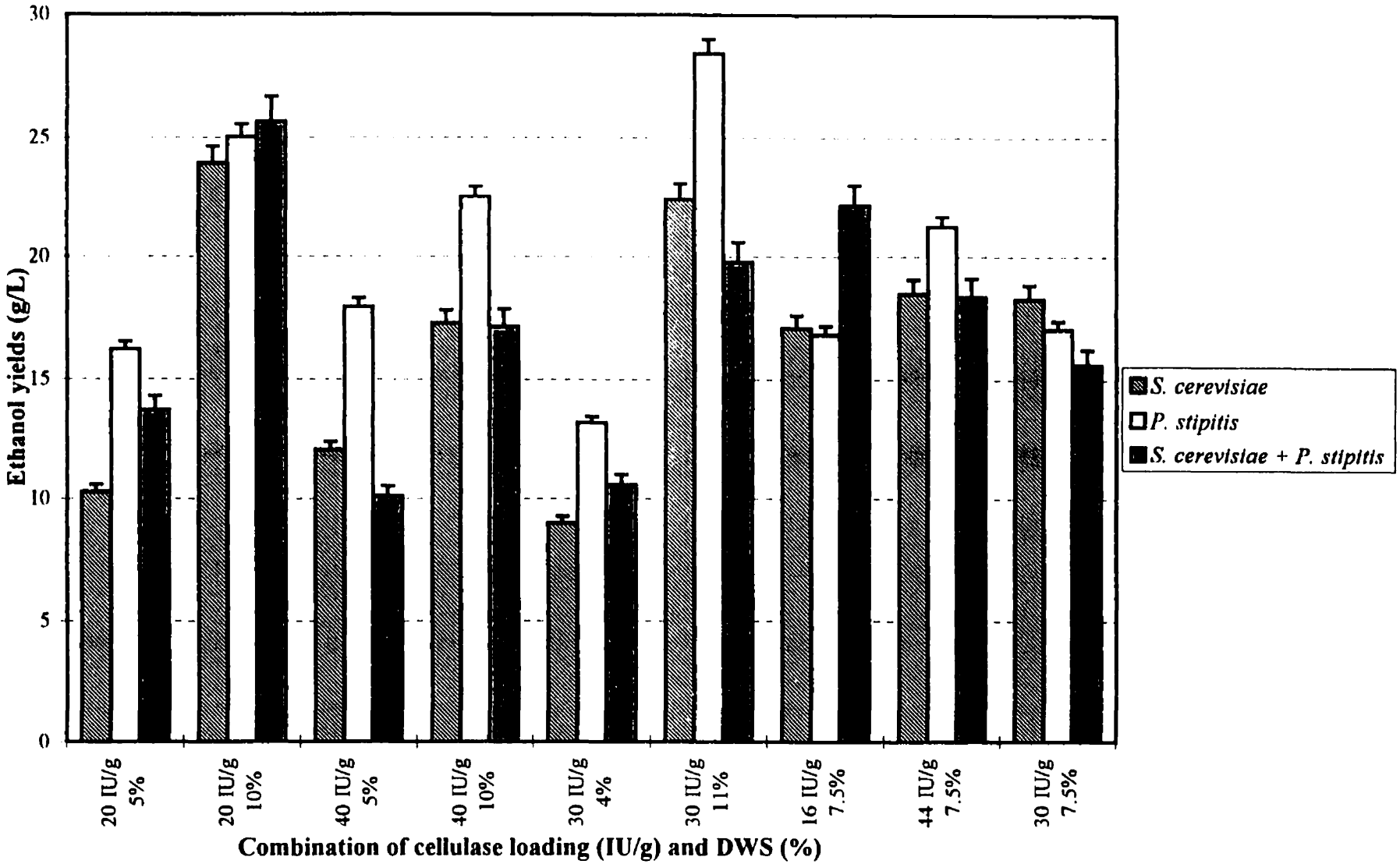


Fig 8-3 Comparison of total reducing sugars to residual reducing sugars after fermentation with different yeast

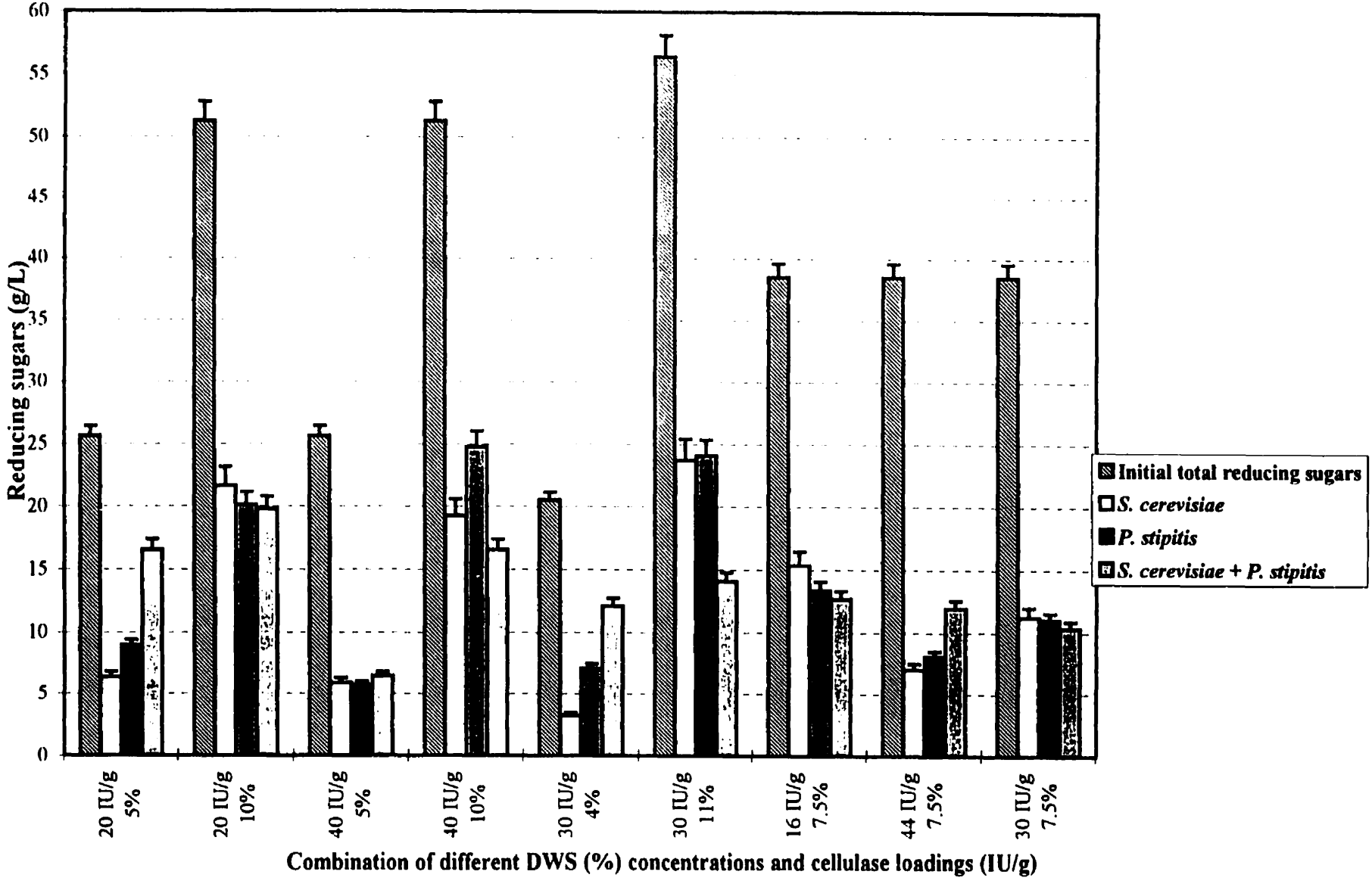


Fig. 8-4 Optimization of delignified wheat straw (DWS) concentration and crude extracted cellulase-system (CEC) loading on ethanol yield with *S. cerevisiae*

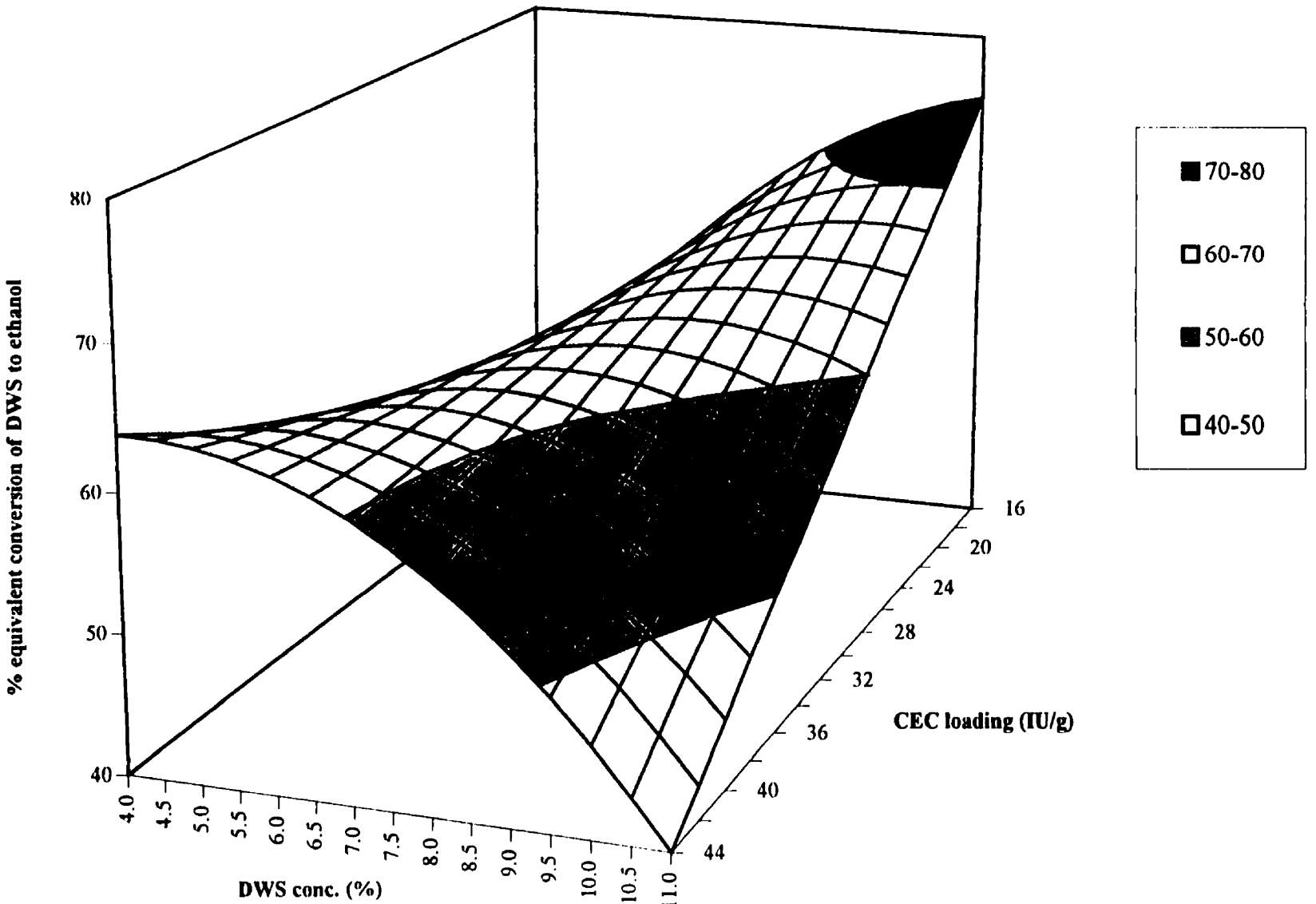


Fig. 8-5 Optimization of delignified wheat straw (DWS) concentration and crude extracted cellulase-system (CEC) loading for ethanol production with *S. stipitis* (Y-7124).

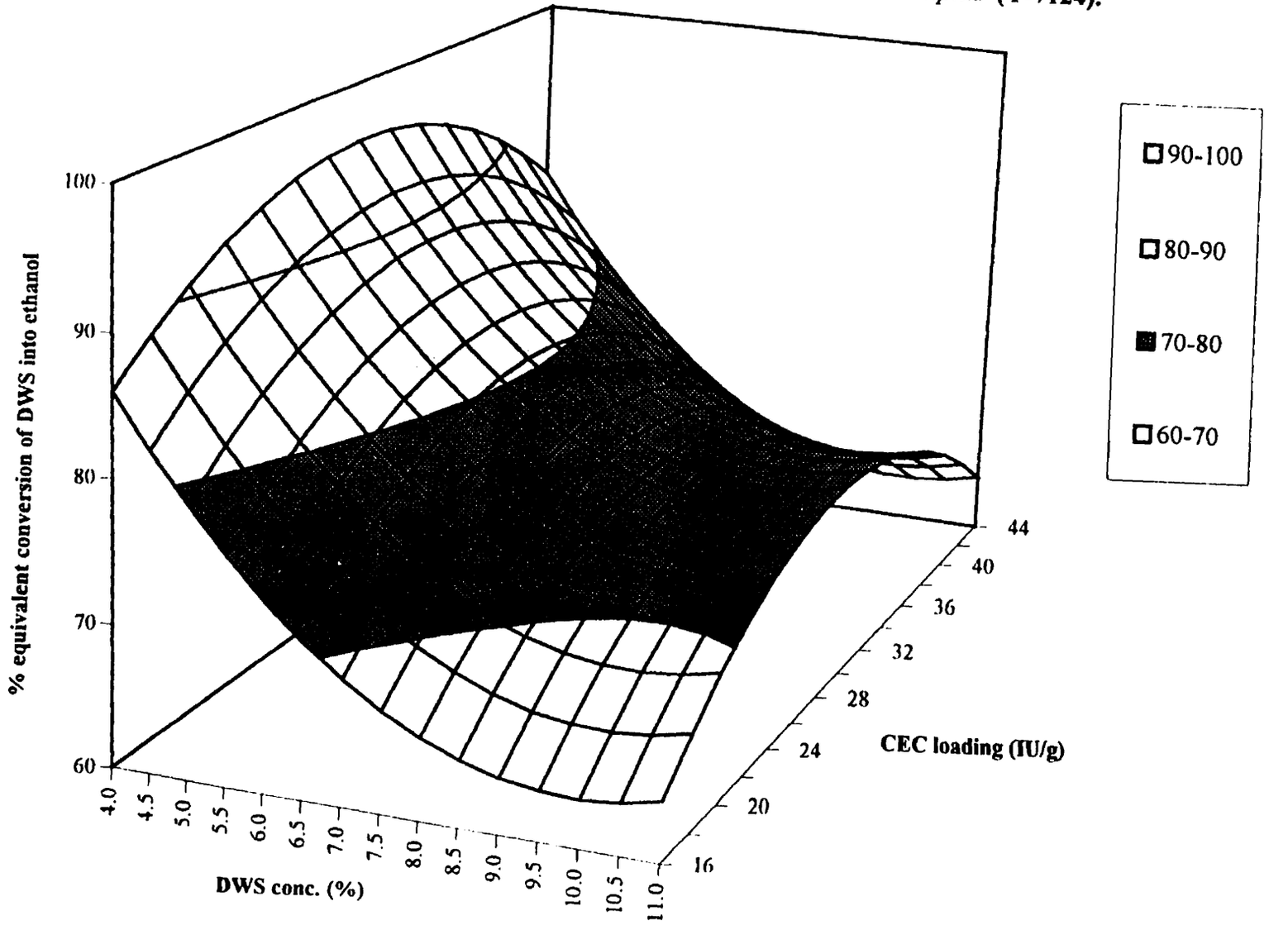


Fig. 8-6 Optimization of delignified wheat straw (DWS) concentration and crude extracted cellulase-system (CEC) loading for ethanol production with a mixture of *S.cerevisiae* and *P. stipitis*

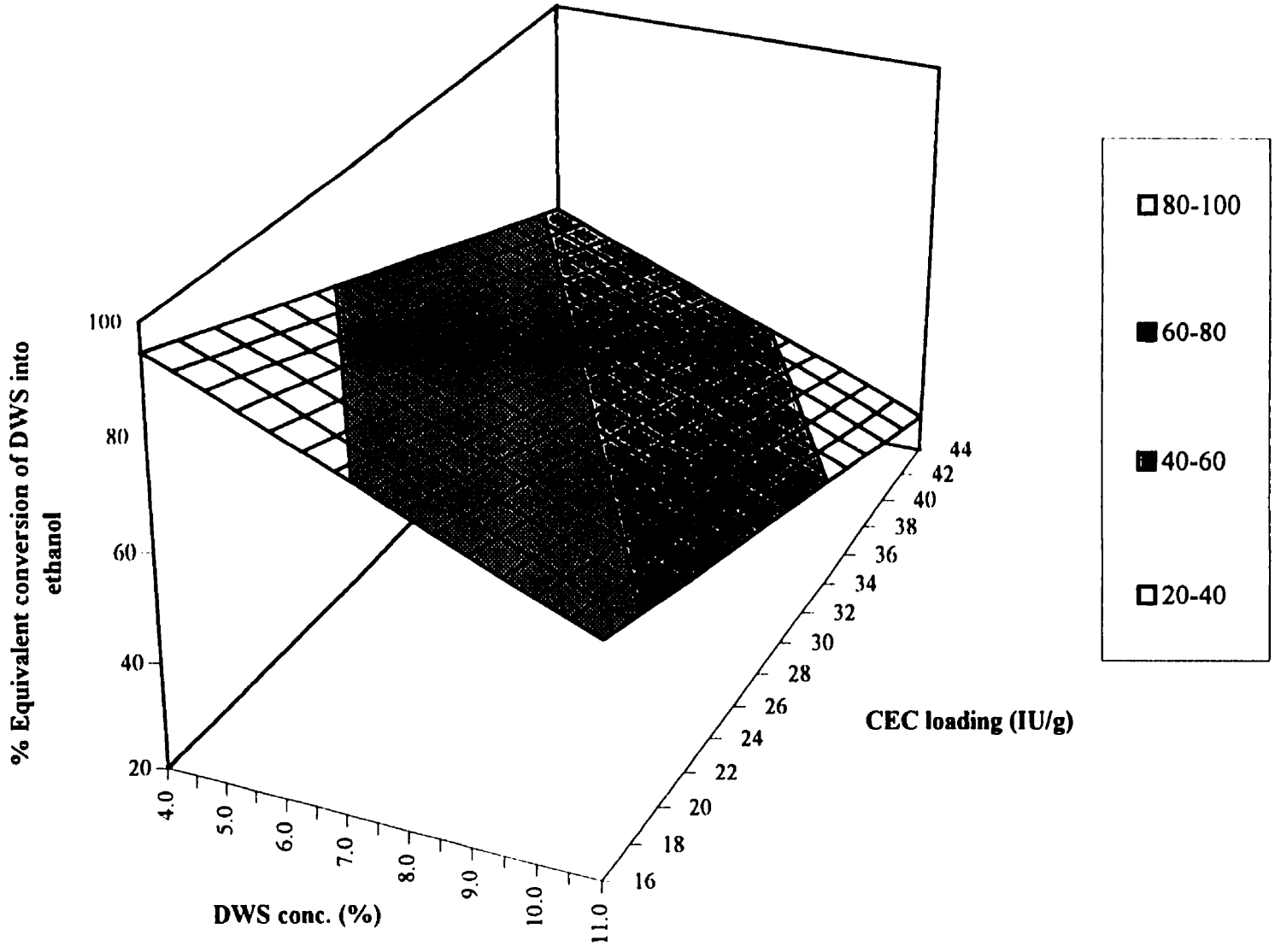


Fig. 8-7 Optimization of delignified wheat straw (DWS) concentration and crude unextracted cellulase-system (CUC) loading on ethanol yield with *S. cerevisiae*

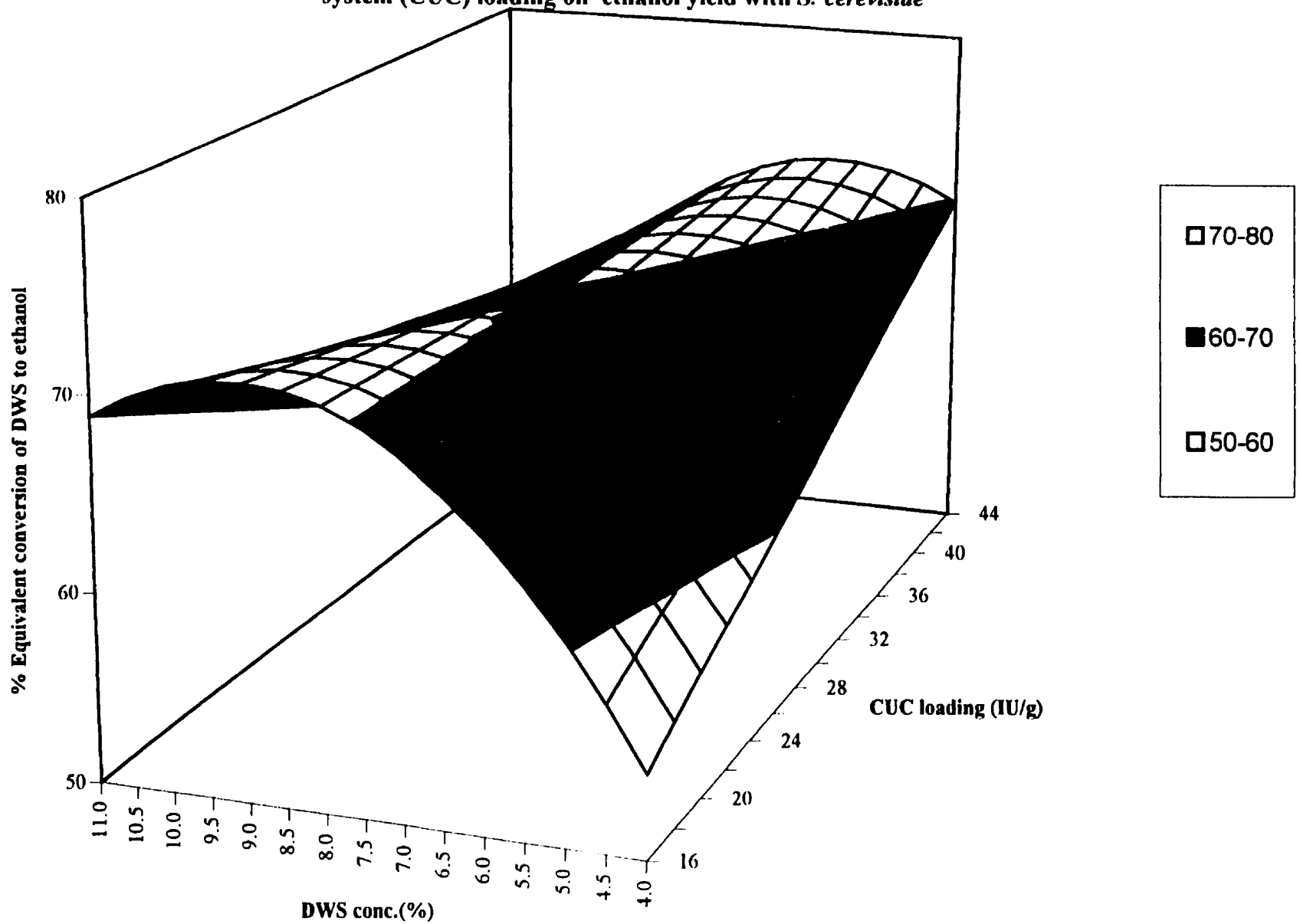


Fig. 8-8 Optimization of delignified wheat straw concentration and crude unextracted cellulase-system (CUC) loading for ethanol production with *P. stipitis* (Y-7124).

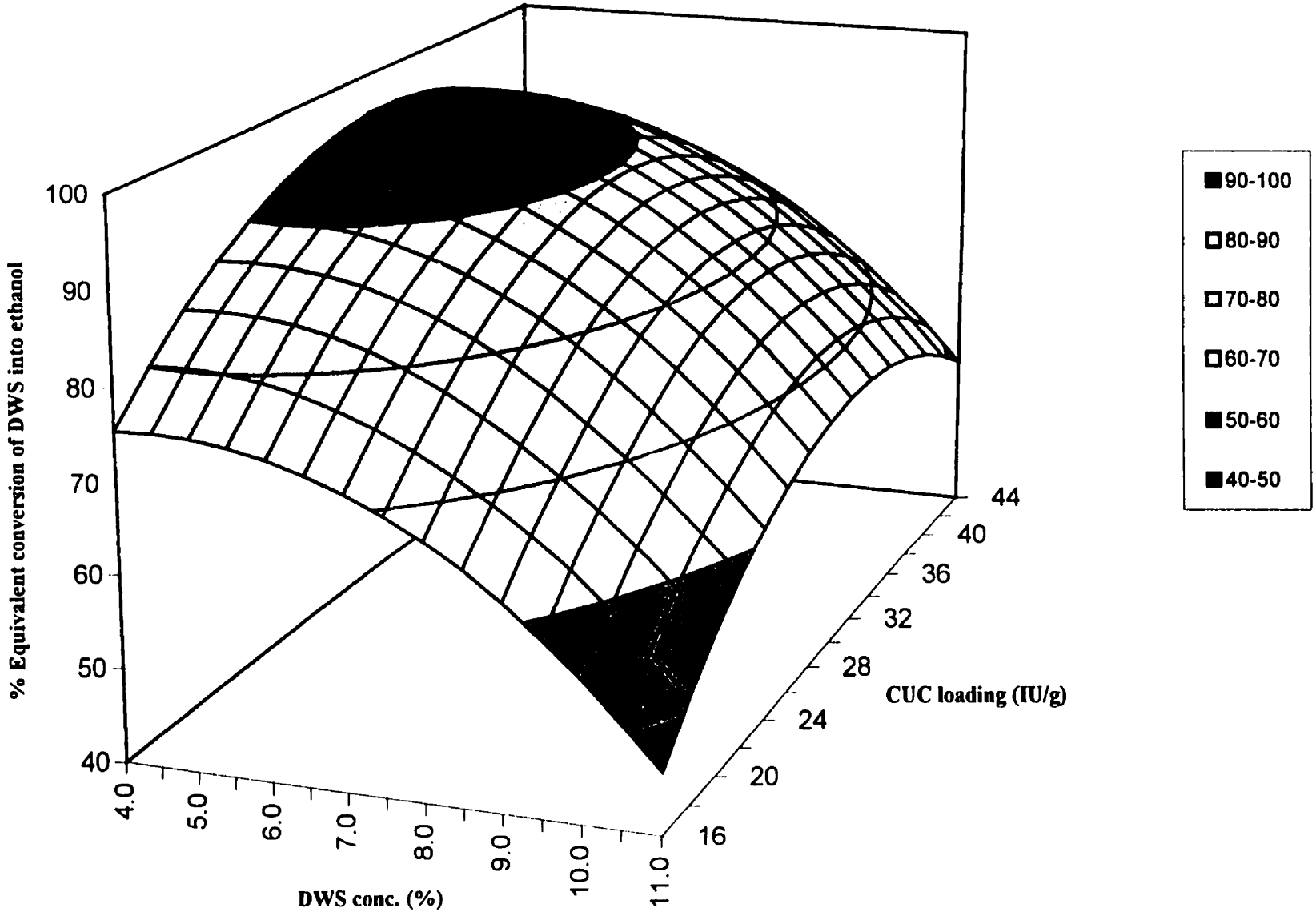
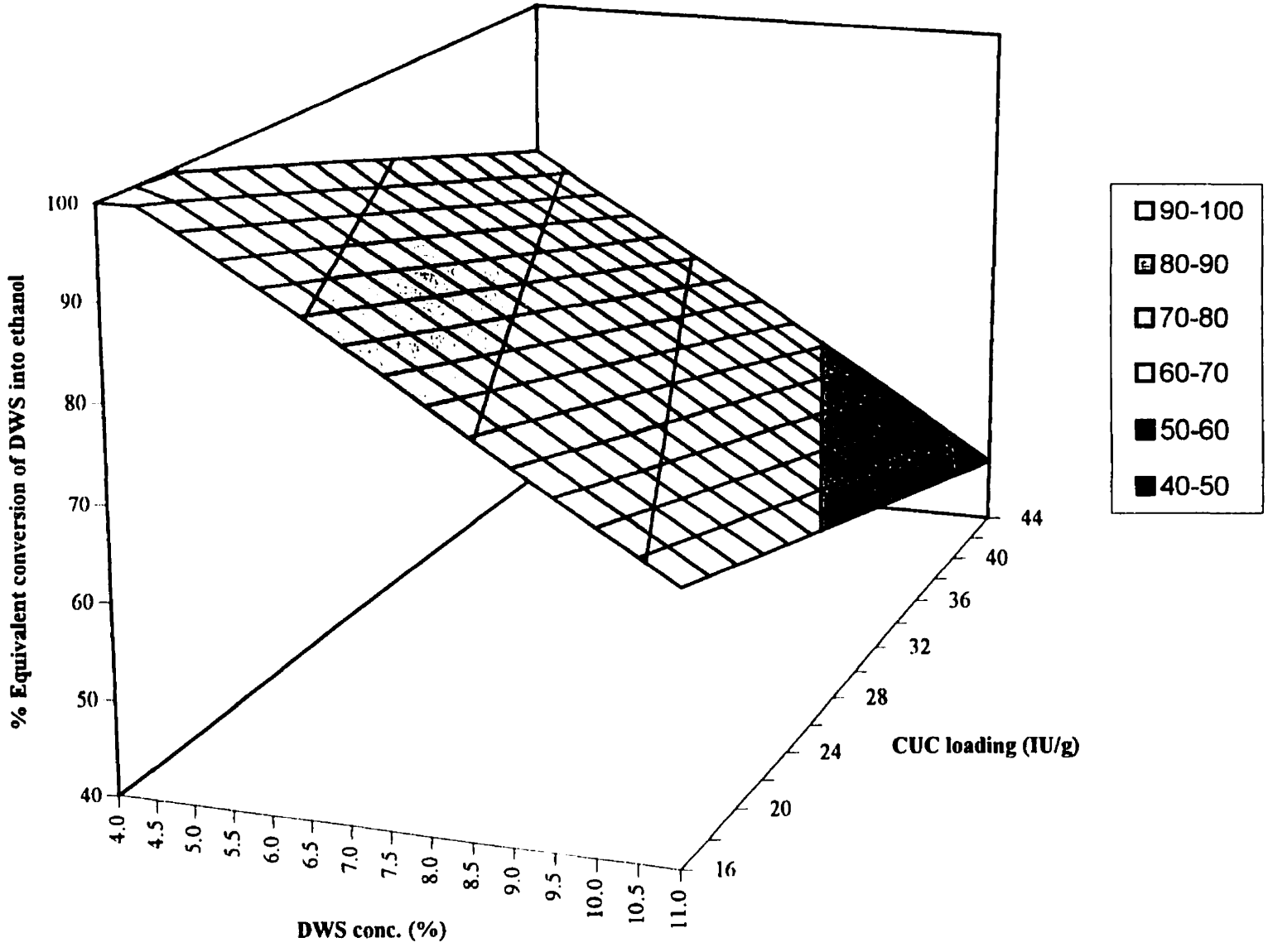


Fig. 8-9 Optimization of delignified wheat straw concentration and crude unextracted cellulase-system (CUC) loading for ethanol production with a mixture of *S. cerevisiae* and *P. stipitis*



CHAPTER 9

GENERAL CONCLUSIONS

The major conclusions which can be drawn from this research are three-fold:

1. Solid-state fermentation (SSF) process using *Trichoderma reesei* QMY-1 and MCG can be utilized for the production of cellulase-systems on lignocelluloses with the following advantages:
   (i) SSF produces higher cellulase-system activities than liquid-state fermentation (LSF) systems
   (ii) The potential for scaling up SSF systems was demonstrated with a prototype Pan-bioreactor
   (iii) Sodium hydroxide pretreated wheat straw is a better substrate for cellulase-system production than steam pretreated wheat straw
   (iv) Cellulase-systems from SSF systems can be used in either the extracted or unextracted form to achieve high cellulose hydrolysis
2. Comparison of the hydrolysis of different pretreated lignocelluloses (delignified wheat straw and steam pretreated wheat straw) and pure cellulose (Avicel, and α-cellulose) with cellulase-systems obtained from *T. reesei* mutants QMY-1 and MCG 80 on wheat straw and commercial cellulases showed that:
   (i) Lignocelluloses were better hydrolyzed than the pure cellulose and delignified wheat straw was the best hydrolyzed substrate

(ii) Crude extracted cellulase-systems from *T. reesei* QMY-1 and MCG 80 had comparable hydrolytic potentials to commercial cellulases

(iii) Unextracted cellulase had higher hydrolytic potential than extracted cellulases and commercial cellulases

(iv) Hydrolysates obtained with crude cellulase-systems were better adapted and utilized for ethanol production by different yeast than hydrolysates obtained from either commercial cellulases, pure glucose or pure xylose

3. Models developed here were capable of predicting

(i) Pretreatment and fermentation conditions (substrate concentration, urea concentration, initial pH etc.) for optimum cellulase-system production

(ii) Hydrolysis conditions (substrate concentration, cellulase loading) to maximize these process parameters in order to achieve over 80% hydrolysis of lignocelluloses.

(iii) Ethanol production conditions (substrate concentration and cellulase loading) with different yeast species (*S. cerevisiae*, *P. stipitis*) to obtain optimum ethanol yields of o.46 g/g substrate.

The overall experimental data generated in this study provided information on the production of cellulase-systems of *T. reesei* on different pretreated wheat straw substrates and their potential for cellulose hydrolysis and subsequent fermentation for ethanol production. From these data, the following conclusions can be derived:

1. Pretreatment of wheat straw with sodium hydroxide suitable for cellulose hydrolysis were found to be detrimental for cellulase production e.g. 0.1 g NaOH /g substrate

produced less than 1.0 IU/mL FPA while lower concentrations of 0.05 NaOH/g substrate yielded up to 6.5 IU/mL FPA.

2. Temperature (25°C and 30°C) and pH profiling (pH 5 and 7) appeared to affect the growth of *T. reesei* mutants but did not affect the production of their cellulase-systems
3. High cellulase filter paper activity (162 IU/g wheat straw) was obtained under SSF compared to 87 IU/mL under LSF from the same substrate and microorganism
4. Wheat straw pretreated and fermented with *T. reesei* MCG 80 under the optimum conditions of 4% NaOH (w/w), sterilization at 121°C for 45 min and pH 6 was capable of producing 9.9 IU/mL (247 IU/g wheat straw) FPA and 6.4 IU/mL (159 IU/g wheat straw) ßGA
5. Unextracted cellulase-system from *T. reesei* MCG 80 and QMY-1 respectively yielded over 90% and 80% hydrolysis of 5% delignified wheat straw while their extracted forms yielded less (56 - 88%).
6. The novel pan-bioreactor evaluated in this study has the potential to upgrade cellulase production in SSF systems but will need further developments to hold higher substrate concentrations without contamination for the duration of the fermentation.
7. Cellulase-systems from mixed cultures of *T. reesei* QMY-1 and *A. phoenicis* were capable of removing all the cellobiose from accumulating during hydrolysis.
8. Ratios of ßGA- to -FPA close to one were demonstrated to be necessary to attain over 80 % cellulose hydrolysis

9. *Pichia stipitis* (Y-7124) and *Sacchromyces cerevisiae* (ATCC 60868) were capable of fermenting over 85% of mixed sugars of hexose and pentose obtained from delignified wheat straw hydrolysates
10. Delignified wheat straw concentration can be optimized between 4 and 7.5% and cellulase loading of 24 IU/g to 30 IU/g to attain 90 - 100 % conversion into ethanol by *P. stipitis*.
11. The maximum ethanol yields obtained from 5% delignified wheat straw under separate hydrolysis and fermentation conditions (120 h) were 15.72 g/L (0.13 g/L/h) with *S cerevisiae* and 25.22 g/L (0.21 g/L/h) with *P. stipitis* while under simultaneous hydrolysis and fermentation conditions (72 h) with same substrate concentration, the ethanol yields were 12.05 g/L (0.17 g/L/h) and 17.95 g/L (0.25 g/L/h) respectively for *S. cerevisiae* and *P. stipitis*

Overall, this study has brought the desire to achieve alternate fuels from agricultural waste closer to home by considering in one package a model substrate for cellulase production, hydrolysis and fermentation for ethanol production by using optimization models that maximize these unit operations. This work should, therefore, stimulate further interest to move from the bench top to commercialization efforts aimed at fuel ethanol production through enzymatic hydrolysis of waste materials.

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IMAGE EVALUATION
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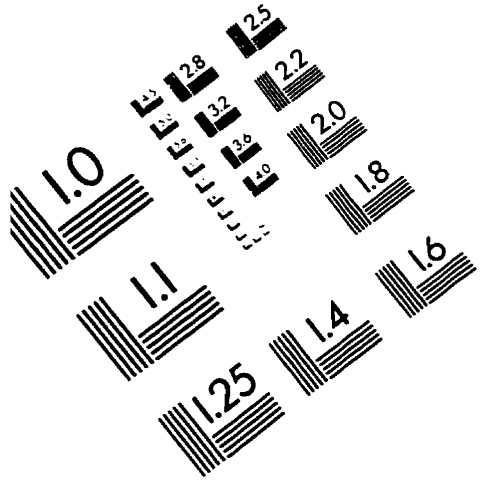

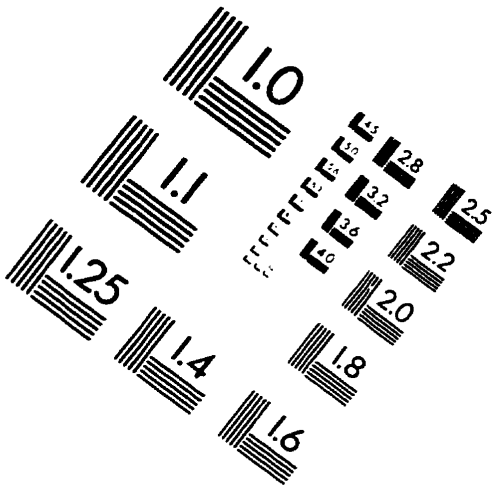

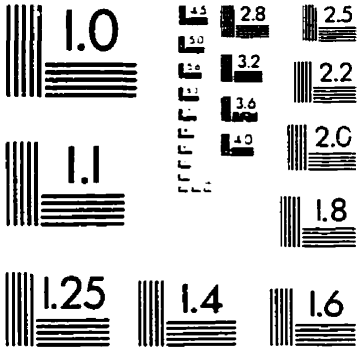

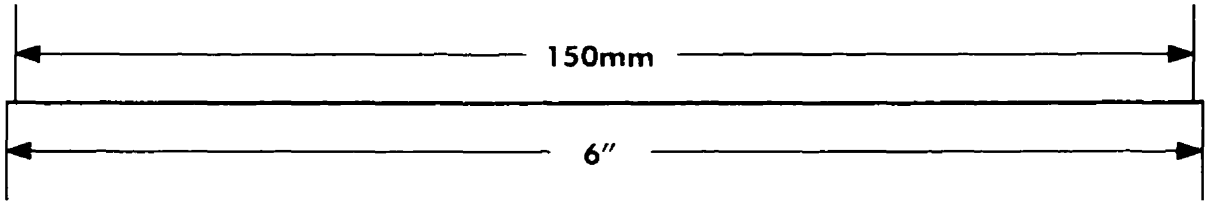

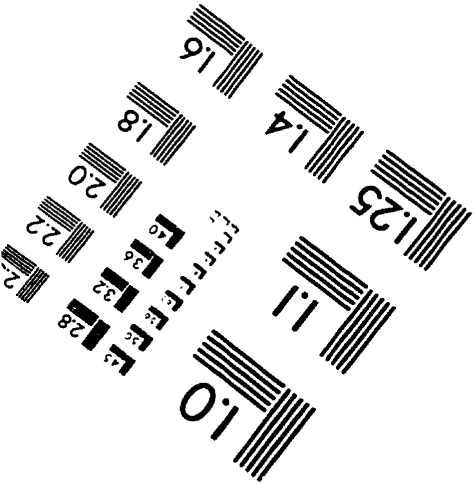

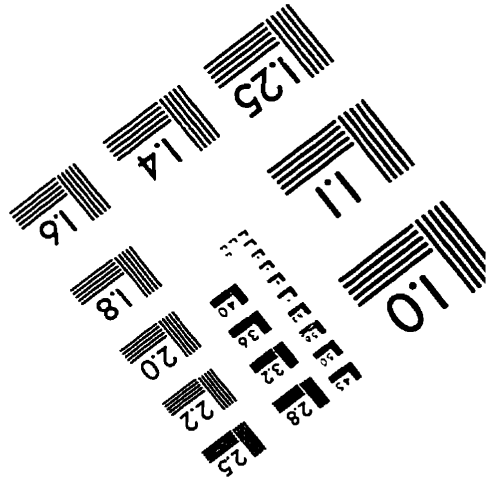

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