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THE ENZYMATIC SACCHARIFICATION OF CANOLA MEAL AND ITS UTILIZATION FOR XYLANASE PRODUCTION BY *TRICHODERMA REESEI*

By

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Abstract

Hemicellulose comprises approximately 20–30% of the lignocellulosic material available as forest and agricultural waste, and represents an abundant and inexpensive source of fermentable sugars. The majority of hemicelluloses are hydrolyzed by the xylanase enzyme system. The bottleneck in industrial scale enzymatic hydrolysis is the high cost of enzyme production. Consequently, to lower production costs, cheaper substrates for the manufacturing of these enzymes are presently being sought. One of the unexplored substrates for xylanase production is canola meal.

Tests made utilizing canola meal as a substrate for the production of xylanase indicate that *Trichoderma reesei* produced this enzyme in similar or better yields from canola meal than from expensive carbon sources such as Solka-floc, cellulose, glucose, lactose, sucrose or purified xylans. The maximum xylanase activity obtained from canola meal was 210 IU/ml in 9–12 days. The effect of culture conditions on xylanase production when canola meal was used as a carbon source was also investigated. The enzyme system produced using canola meal also contained a higher proportion of acetyl-xylan esterase, cellulase, and xylosidase activities, most of which are required for synergistic action and hydrolysis of complex materials. This system was more or equally efficient in hydrolyzing canola meal, corn cobs, corn and wheat bran, straw, and larchwood xylan to fermentable sugars as compared to that produced using Solka-floc. The physicochemical properties, pH, temperature optima and thermal stability of the enzyme system produced using canola meal and Solka-floc as carbon sources were

compared and determined to be essentially the same.

The enzymatic saccharification of canola meal was also investigated. Autoclaving pretreatment was necessary for the enzymatic saccharification of canola meal by enzyme preparations from *T. reesei* as well as by commercially available hemicellulase and multienzyme preparations. These enzyme preparations hydrolyzed over 20% (w/w) of pretreated canola meal, which constitutes over 70% saccharification of the total polysaccharides present in the canola meal. A higher extent of saccharification was acheived at 50°C relative to 37°C. The results show that saccharification of canola meal is mainly brought about by hemicellulases capable of degrading arabinogalactan, arabinoglucan, galactan and galactomannan, while cellulase and xylanase play a minor role. The hemicellulases were found to be more stable at 50°C than cellulases or xylanase. This autoclaving pretreatment also released water soluble polysaccharides consisting mainly of arabinnose and glucose. *T. reesei* was unable to produce enzymes capable of hydrolyzing these polysaccharides when cultivated on canola meal as substrate.

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Chapter 1

Introduction

Lignocellulosic materials represent an abundant, inexpensive and rapidly renewable source of feed and chemical products which are available through bioconversion. Canadian resources of lignocellulose include vast quantities of readily available forest and agricultural waste. Lignocellulose is composed of 40–60% cellulose, 20–30% hemicellulose and 15–30% lignin (Dekker, 1983). Until recently, most research has investigated cellulose degradation (Ryu and Mandels, 1980), but hemicellulose degradation is also necessary for economic feasibilty of the bioconversion process (Suh *et al.*, 1988). Consequently, present work is now also focussing on the enzymatic degradation of hemicellulose.

Hemicellulose is composed of a variety of polysaccharides, the most common of which is xylan (Dekker, 1983). Xylan hydrolysis has not been as extensively studied as that of cellulose because glucose, (the product of cellulose breakdown) is more valuable than xylose and the other heterogeneous products resulting from xylan degradation (Reilly, 1982). However, after cellulose, xylan is the next most abundant renewable polysaccharide in nature (Biely, 1985). If xylan is not concurrently converted with cellulose, the cost of raw materials would become so high due to the large amounts of unused xylan that the cellulose breakdown products would not be economically

competitive. Consequently, to ensure the success of industrial utilization of cellulosic residues, hydrolysis of xylan is essential (Reilly, 1982).

The industrial scale enzymatic hydrolysis of lignocellulosic materials will use the following stages; pretreatment of substrate, enzyme production, enzymatic saccharification of the pretreated substrate, fermentation of the hydrolysis products and enzyme recovery. The major bottleneck in the industrial scale enzymatic hydrolysis is the high cost of enzyme production. Enzyme production costs alone account for more than half of the total processing costs (Deshpande and Eriksson, 1984; Dekker, 1983). Therefore in order to enable the industrial conversion of lignocellulosic materials to become economically feasible the availability of bulk inexpensive and highly active enzyme preparations is imperative.

One of the major reasons why enzyme production is so expensive is due to the high cost of substrates which are presently being used to produce these enzymes. With respect to xylanases, these enzymes are presently produced from processed or refined substrates such as xylans. These substrates are relatively expensive for industrial scale production. Consequently, to lower the production costs, cheaper substrates for the manufacturing of these enzymes are presently being sought (Warzywoda *et al.*, 1983; Tangnu *et al.*, 1981; Sarker and Prabhu, 1983). One of the unexplored substrates for xylanase production is canola meal.

Rapeseed and canola, are the only oil seeds that can be grown on a commercial scale in the cooler areas of the world such as Canada, China, Northern Europe, and the Indian subcontinent (Butler *et al.*, 1982; Meikle and Co., 1986). The oil processed from canola is much lower in harmful materials such as erucic acid and glucosinolate than rapeseed oil, consequently its production has increasd by over 300 percent in the last decade (Harris, 1987). Canola meal is a by-product after oil extraction from canola. The present use for canola meal is limited to animal feed. However, canola seed coat has been shown to depress protein digestability and reduce bioavailability of Cu and Zn in animal diet (Ward and Reichart, 1986). It has been also shown that

canola meal causes liver damage in poultry and taints eggs (Butler *et al.*, 1982). Since canola meal is available in vast quantities in Canada, and its present use as animal feed is restricted because it is toxic in large doses, finding an alternative use for canola meal is an attractive proposition.

The purpose of this study was to investigate the possibility of producing xylanases by *T. reesei* using canola meal as a carbon source. This investigation was to be carried out by comparing xylanse production, types of enzymes produced, physicochemical properties and applications of the enzyme preparations produced using canola meal with that of more expensive substrates. This study also investigates the enzymatic saccharification of canola meal, and determines the nature of enzymes involved in this process.

Chapter 2

Literature Review

In addition to cellulose degradation, hemicellulose degradation is also necessary for economic feasibility of the lignocellulosic bioconversion process (Suh *et al.*, 1988). Consequently, present research is now also focusing on the enzymatic degradation of hemicellulose. The most common polysaccharide found in hemicellulose is xylan (Dekker, 1983), and after cellulose, it is the next most abundant renewable polysaccharide in nature (Biely, 1985). Therefore, to ensure the success of industrial utilization of cellulosic residues, hydrolysis of xylan by an economically feasible process is essential (Reilly, 1982).

The scope of this chapter includes discussion about xylan and the xylanase enzyme system, reasons for the choice of microorganism in this study, general information about canola meal, and the future of lignocellulose conversion.

2.1 Xylan

In order to facilitate bioconversion of lignocellulosic materials, many details have yet to be elucidated concerning lignocellulose structure and the mechanisms of its enzymatic saccharification. The complex structure of lignocellulose results in the need for a very

complex enzyme system for its complete hydrolysis. This study focuses on the xylanase enzyme system, one of the enzyme systems necessary for degradation of xylan, the most common polysaccharide found in hemicellulose.

Before continuing with this discussion it is important to mention that one method which is presently being used on an industrial scale to hydrolyse lignocellulosic materials is acid hyrolysis. This method uses either dilute or concentrated sulfuric and hydrochloric acids or hydrofluoric acid, and is quick and easy. It is also presently less expensive than enzymatic hydrolysis. If conditions are sufficiently mild xylan can be hydrolyzed in the presence of cellulose without significant attack on cellulose. The disadvantage of acid hydrolysis is because it is not specific a variety of products are obtained and sugars are lost due to formation of reversion products. The use of acids also causes corrosion problems as well as adverse environmental effects due to emissions from the acid hydrolysis process and additional costs associated with using charcoal to treat the hydrolysate to remove color and the smokey aroma (Reilly, 1982). The advantages of using enzymatic hydrolysis instead of acid hydrolysis are: enzymes are non-corrovise and specific in their action. They are environmentally acceptable and efficient re-use of enzymes would make this process more economically feasible (Reilly, 1982).

Lignocellulose is composed of 40–60% cellulose, 20–30% hemicellulose and 15–30% lignin (Dekker, 1983). Cellulose is a linear high molecular weight polymer of glucose. In most plant material the cellulose fibres are cemented together and bonded to lignin and hemicellulose. Lignin is a high molecular weight amorphous, aromatic "plastic", while hemicellulose is a low molecular weight, generally amorphous polysaccharide (Thompson, 1983). The five main sugar residues which comprise hemicellulose are D–xylose, D–glucose, D–galactose, L–arabinose and 4–O–methyl–D–glucuronic acid (Woodward, 1984).

Xylan structure varies, it can be in the form of linear 1,4–β–linked polyxylose chains to highly branched heteropolysaccharides (Figure 2.1). The main chain of xylan

is composed of repeating units of the five carbon sugar D-xylose. The branches consist of L-arabinofuranose (L-arabinose) linked to the O-3 positions of the D-xylose residues and D-glucuronic acid or 4-O-methyl-D-glucuronic acid linked to the O-2 position. The degree of branching depends on the source. Some of the xylose units may also be acetylated, for example birch xylan (Biely, 1985). The heterogeneous β-1,4-xylan polysaccharide structure is found in the cell walls of all land plants and in almost all plant parts. Xylan appears to be a major interface between lignin and carbohydrates in secondary plant cell walls (Wong *et al.*, 1988).

Polymeric xylan is too large to enter microbial cells, therefore it must be degraded extracellularly. Consequently, enzymes such as xylanases are secreted by microorganisms into the surrounding medium, and the monomeric degradation products are ingested by the cells (Biely, 1985). Xylosidic linkages in lignocellulose are not all the same and equally accessible to xylanolytic enzymes. The production of an enzyme complex containing enzymes each with special functions is one way that microorganisms use to maximize hemicellulose hydrolyis (Wong *et al.*, 1988). The complete breakdown of branched acetyl xylan requires the synergistic action of many hydrolytic enzymes (Figure 2.1), (Biely, 1985). The hydrolysis of the β-1,4-linked D-xylose backbone is accomplished by β-1,4-xylanases which are referred to as D-xylanases (or xylanases) and the β-xylosidases (or xylosidases). Xylanases are classified as either exo-β-xylanases which cleave the backbone from the ends of the chain, or endo-β-xylanases which cleave the backbone internally. Like the exo-β-xylanases, the β-xylosidases are also involved in endwise attack of xylooligosaccharides, but they generally attack shorter chains and the β-xylosidase product configuration is not inverted (Woodward, 1984). Relatively little is known about the α-L-arabinofuranosidase and α-glucuronidase enzymes (Biely, 1985). Acetyl-xylan esterases are necessary for the removal of acetyl groups from acetylated xylan thereby producing acetic acid. Esterases and xylanases are both essential for acetyl xylan hydrolysis and without them xylan hydrolysis is impeded (Biely *et al.*, 1988). The details of the mechanism of xylan degradation are

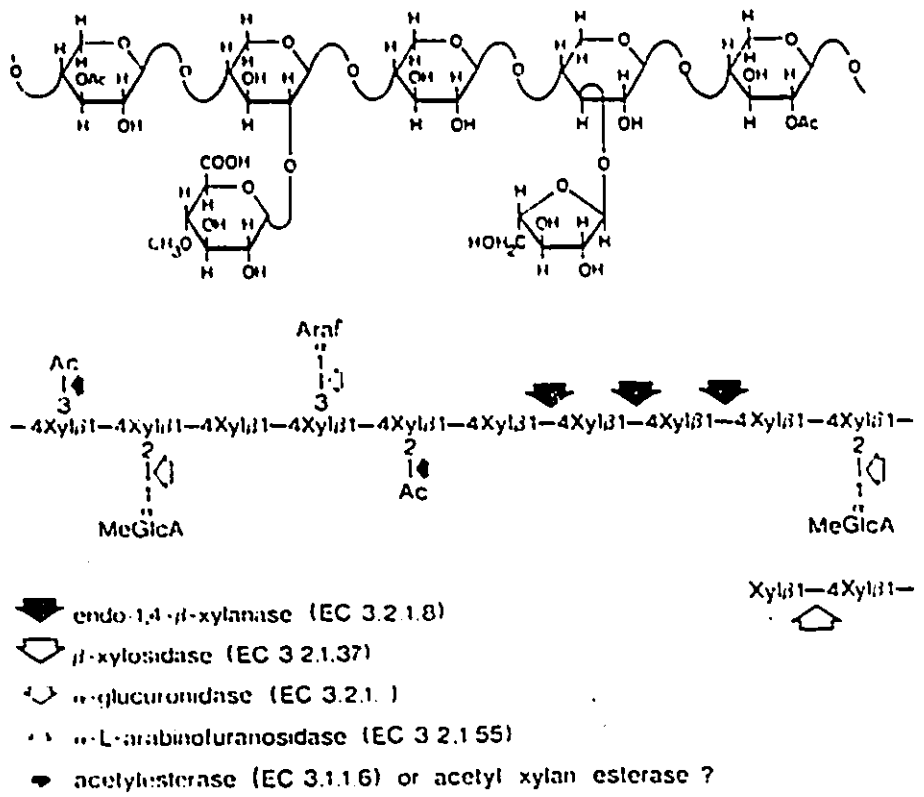

Figure 2.1: Example of Plant Xylan Structure and its Attack by Microbial Xylanolytic Enzymes. The Fragment in the Upper Part of the Figure is Comprised of Five D–Xylose Units. Ac, acetyl group; Araf, L–arabinfuranose; MeGlcA, 4–O–methyl–D–glucuronic acid; Xyl, D–xylose

not yet fully understood (Dekker, 1985), and further studies are needed to completely understand the microbial degradation of xylan (Biely, 1985).

Multiple xylanases have been reported in many microrganisms. (Dekker, 1983). For example, three major and ten minor xylanases are present in Cellulysin, a commercial enzyme preparation from *T. viride* (Biely, 1985). Minor xylanases may have functions which are not required in large quantities for example in the hydrolysis of linkages which do not occur very often. Some of the multiple enzymes may also be allozymes, products of different alleles of the same gene. Wong *et al.* (1986) found that the xylanase multiplicity in *T. harzianum* is necessary for effective hydrolysis of xylan in complex substrates. The enzymes they characterized are not redundant enzymes but each makes a significant and unique contribution to the xylanolytic system of the fungus.

A knowledge of some of the physicochemical properties of xylanase is necessary when these enzymes are used in the saccharification of substrates to ensure that they are operating under conditions where they are most active (Dekker, 1983). The temperature, duration of incubation, pH, and nature of substrate all influence the efficiency of enzymatic hydrolysis (Saddler *et al.*, 1985). The properties to be reported here are the net result of the combination of the enzyme components produced by *T. reesei* when it was grown on various carbon sources, and are not due to a single enzyme. For example, xylanase is a complex variety of enzymes such as D-xylanase, β-xylosidase and acetyl-xylan esterase. The physicochemical properties are representative of all the enzyme activities present (Chaudhary and Tauro, 1986). Often these enzyme components may differ in their physicochemical properties without showing any significant differences in the hydrolytic activities to insoluble xylans (Mitsuishi *et al.*, 1988) No attempts were made to separate the enzyme components.

The applications of the xylanase enzyme system are extremely varied. Xylanases play an important role in plant litter decomposition and biomass turnover. The main function of xylanases in biodegradation is to provide a source of metabolizable energy

in the form of carbonaceous substrates which are easy to assimilate (Woodward, 1984) . thereby maintaining the flow of carbon in the carbon cycle (Wong *et al.*, 1988).

It is hoped that eventually the use of plant materials for the production of fuels and chemicals can at least partially replace the use of fossil fuels. Xylanases are necessary for the hydrolysis of xylan to products such as xylose which can then be converted to ethanol and butanol by microorganisms such as yeasts (Woodward, 1984) and fungi (Linko *et al.*, 1982). The saccharification and fermentation can be carried out as separate processes or as a single step process, with simultaneous saccharification and conversion (Ladisch *et al.*, 1983).

Fungal treatment has been suggested for paper manufacturing because it can reduce the energy cost of refining pulp. The use of purified xylanases may reduce pulp treatment time and could also be used for the removal of contaminating hemicellulose components from high-grade cellulose pulps (Tan *et al.*, 1987). A range of desirable pulp characteristics may also be obtained by using xylanases to selectively hydrolyse certain xylan components. Total xylan removal from fibre is not necessarily desirable because xylan contributes significantly to fibre strength and therefore paper quality (Wong *et al.*, 1988). In addition, wastes from the pulp and paper industry contain large amounts of chemicals and the reversion products of hemicellulose in a moist form which cannot be burnt readily and are therefore costly to dispose. In order to reduce these costs and environmental pollution, the hemicellulose fraction can be converted microbially to ethanol and other useful products (Lemmel *et al.*, 1986).

Presently, fibre liberation from plant fibre sources such as flax is affected by retting which is the removal of binding material in plant tissues using enzymes produced in situ by microorganisms. It is hoped that replacement of slow, natural retting processes by treatment with artificial mixtures of enzymes will become a rapid new method for fibre liberation technology (Biely, 1985).

Xylanases are presently used in combination with pectinases for the clarification of juices and for the liquification of fruits and vegetables (Biely, 1985). Xylanases are

also used in preparations of dextran for food thickening. Partial xylan hydrolysis of animal feed may also be used to improve cellulose accessibility to ruminal digestion and therefore improve the nutritional value of the food. This is of interest in the use of canola meal as a feed as well. The application of xylanases will depend very greatly on their availability at a reasonable cost (Wong *et al.*, 1988). The use of agricultural wastes as inexpensive substrates to lower the cost of enzyme production is presently being investigated by a variety of workers. This study parallels the work of researchers who are presently using a variety of agricultural substrates for xylanase enzyme production by *T. reesei*.

The use of a variety of microorganisms and substrates for the production of xylanases has been investigated by many workers. Most work on bacterial production of xylanases is limited to the genera *Bacillus* and *Streptomyces* (Dekker, 1985). After surveying twenty-five *Bacillus* strains for xylanase production Bernier *et al.* (1983) selected *B. subtilis* PAP115 for further investigation. They used a 14 litre fermenter for large-scale enzyme production. The medium was composed of the essential nutrients as well as 10% (w/v) of the carbon source xylan. After 3 days of cultivation at an aeration rate of 1.2 l/min and an agitation rate of 200 rpm at 30°C, the culture was harvested and stored at 4°C. The activity acheived was 0.8 IU/ml. Okazaki *et al.* (1984) studied xylanase production using four strains (W1–W4) of alkalophilic thermophilic *Bacillus* spp. The cultures were grown in L-shaped test tubes at pH 10, at 45°C for 48h using carbon sources such as xylan, xylose and glucose. In cultures containing 1% glucose and xylose activities did not surpass 36 IU/ml. In the case of 1% larchwood xylan the highest xylanase activity obtained was 112 IU/ml by W3. Other workers (Nakajima *et al.*, 1984) have found that *Streptomyces* sp. KT-23 was able to use 0.05% rice-straw arabinoxylan as the sole carbon source in a liquid medium at 30°C for 48 h with shaking to produce less than 10 IU/ml of xylanase activity.

Xylanases of fungal origin have been described in much detail and are well characterized. Yeasts are recognized as xylanase producers. Xylanase production appears to

be found mainly in the genera *Aureobasidium, Cryptococcus* and *Trichosporon* (Dekker, 1985). When glucose is used as the carbon source for growth of the yeast *Cryptococcus albidus* low levels of mainly cell-wall-associated endoxylanase was produced, although the enzyme can be produced constitutively it can also be produced by growth on xylan (Biely *et al.*, 1980).

Some of the less commonly studied fungi include the thermophilic fungus *Thielavia terrestros* (Merchant *et al.*, 1988) and the fungus *Sporotrichyum thermophile* (Margaritis *et al.*, 1983). Merchant *et al.* (1988) studied the effect of varying fermentation pH and temperature on extracellular xylanase production in a stirred tank bioreactor. They found that using 1% Solka-floc resulted in a maxiumum xylanase activity of 18.8 IU/ml which was obtained when the temperature was controlled at 48°C and the initial pH was 4.0. Margaritis *et al.* (1983) found that when wheat straw was used as a carbon source, *S. thermophile* was able to produce extracellular xylanase. In this case, *S. thermophile* was grown in a 12 l fermentor at 48° with aeration for 72 hours. The medium used was a nutrient medium containing wheat straw. The xylanase enzyme activity achieved after 72 hours of fermenation was 7.8 IU/ml.

The *Aspergillus* genus of fungi is also being investigated for its ability to produce xylanse. For example Gokhale *et al.* (1986) found that *A. niger* NCIM 1207 was able to produce 10.0, 5.1, and 26.5 IU/ml of xylanase activity when it was grown on 4% xylan, 4% wheat bran, and 3% cellulose respectively using the Reese and Mandels medium. Chen *et al.* (1986) have also shown that *A. terreus* A07 is capable of xylanase production.

This survey of the literature revealed that although investigation into fungal xylanase production is the most extensive, by far the majority of work studies xylanase production by the genus *Trichoderma.* Some species which are presently being studied include *T. viride* (Gibson and Cleary, 1987), and *T. koningii* (Wood and McCrae, 1986). The species which will be discussed in more detail here are *T. harzianum* and *T. reesei.*

Saddler *et al.* (1985) were able to achieve xylanase activities of 230 and 30 IU/ml using 1% larchwood xylan and 1% Solka-floc respectively as carbon sources using *T. reesei* RUTC30. The cultures were grown in shake flasks using Vogel's medium. Under the same experimental conditions, *T. harzianum* E58 was able to acheive xylanase activities of 434 and 240 IU/ml using 1% Solka-floc and larchwood xylan respectively. In addition, *T. reesei* RUTC30 when grown on steam exploded aspen (SED) wood and SED extracted with water produced 218 and 140 IU/ml of xylanase respectively while under the same conditions, *T. harzianum* E58 produced activities of 350 and 450 IU/ml of xylanase respectively. Senior *et al.* (1988) studied the use of cellulase-free xylanase preparations from *T. harzanium* for degradation of xylan in pulp.

Chaudhary and Tauro (1986) found that *T. reesei* QM9414 produced little or no xylanase in a medium containing 1% glucose as a carbon source. The cultures were grown in shake flasks and incubated on a rotary shaker at 250 rpm. Using 1% larchwood xylan as a carbon source they were able to attain a xylanase activity of 9.2 IU/ml after 5 days.

Robison (1984) used *T. reesei* RUT C30 to produce 130 IU/ml of xylanase activity using 1% larchwwod xylan as a carbon source. The fermentation was carried out at 28°C in a 7 l fermenter using the Natick media. The pH was maintained above 3.0 with NaOH. The oxygen controller regulated air bubbling at a rate such that dissolved O_2 levels of $\pm 1\%$ saturation could be maintained.

The results of Tangnu *et al.* (1981) showed that *T. reesei* RUTC30 was able to produce a xylanase activity of 114 IU/ml using 1% Solka-floc as a carbon source. The fermentations were carried out in a 14 l fermentor with an operation volume of 10 l. The dissolved oxygen was controlled at a level greater than 20% of the medium saturation value. The temperature was maintained at 25°C and the pH was maintained at greater than or equal to 5.0. The medium used was a modified version of the Mandels and Weber medium.

Szczodrak (1988) investigated xylanase production by *T. reesei* F-522 using hy-

drolyzed wheat straw pulp at a concentration of 1.5% as a carbon source. The enzyme production media was that of Mandels and Weber (Szczodrak, 1988), with proteose peptone and Tween 80 supplements. The culture was run for 7 days in shake flasks at 28°C at 220 rpm. The results of the study indicated that 11.4 IU/ml of xylanase activity was acheived after 8 days.

Kraft paper mill sludge can also be used as a substrate for xylanase production (Royer and Nakas, 1987). The results showed that *T. reesei* DAOM 167654 and *T. longibrachiatum* produced xylanase activities of 8.7 and 35.1 IU/ml after 7 days of growth on this substrate. The media used for this study was a modified version of Park's media. The experiment was run in shake flasks at 28°C at 80 rpm.

In 1988, Ghosh and Deb looked at the use of xylan, rice straw, peanut shell, Sarkanda leaves and corn cob as substates for xylanse production by *T. reesei*, they acheived xylanase activities of 7.3, 12.0, 1.5, 7.0, and 7.2 IU/ml respectively. They obtained a maximum xylanase activity of 34 IU/ml using rice straw after 12 days. The cultures were grown for 24 h in an orbital shaker at 30°C and 250 rpm. The media was modified Vogel's salt solution, with 1% of the various carbon sources and 0.2% Tween 80.

Research is now also beginning to investigate the production of hydrolytic enzymes by *T. reesei* using solid state fermentation (Atev, 1986). Atev has found the *T. reesei* is able to grow under solid-state fermentation conditions using lignocellulose substrates such as alfalfa cake, wheat bran, and wheat straw, as substrates for enzyme biosynthesis. Xylanase activities as high was 390 IU/g of substrate were acheived.

For the enzymatic hydrolysis of lignocellulose to be economically feasible, the hydrolysis of both cellulose and hemicellulose is essential. In nature, cellulose is generally associated with hemicellulose, lignin and other polymers which make its enzymatic hydrolysis difficult (Durand *et al.*, 1984). The lignin present in the biomass forms barriers which block the penetration by polysaccharide digesting enzymes (Chahal, 1982). Since this study focuses on the degradation of hemicellulose composed of xylan, the

logical choice of microrganism is one which has both cellulase and xylanase activites and would be even more effective if it also contained as wide as possible a variety of secondary carbohydrolyases and lytic enzymes.

As discussed earlier xylanases are found in a variety of microorganisms. *Trichoderma reesei* has presently received a lot of attention because it is the organism with the highest potential for large scale cellulase production. *Trichoderma reesei* RUT C30 NRRL 11400 was used in this study because in addition to being a hyper cellulase producing mutant, it produces xylanases in substantial amounts, as well as a broad spectrum of enzymes necessary for the hydrolysis of plant cell-wall polysaccharides and plant gums. Since *T. reesei* is able to produce an enzyme system capable of hydrolyzing cellulose, glucan and hemicelluloses the use of these enzymes produced by a single microorganism is more feasible economically for the hydrolysis of hemicelluloses (Khan *et al.*, 1989*a*).

Trichoderma reesei RUT C30 has been extensively studied for cellulase synthesis (Ryu and Mandels, 1980). Since, in this microorganism, the synthesis and the control of xylanase is independent of cellulose formation (Chaudhary and Tauro, 1986; Hrmová *et al.*, 1986), the work carried out on cellulase production is not applicable for the synthesis of xylanase.

If xylanases are to be used commercially for the hydrolysis of biomass sources there are still many areas of research to be pursued. One such area is the use of recombinant DNA technology for the production of hyper-producing enzyme microbial strains. Others include the development of more thermally stable xylanases and method for easy recovery and reuse of xylans (Woodward, 1984). Many xylanolytic organisms possess the metablic pathways necessary for single step conversion of xylan and xylose to ethanol; often the depolymerization reaction is rate-limiting in xylan fermenations, therefore two-step processes or mixed culture with two different microorganisms will also have to be considered.

2.2 Canola

It has been shown that when the same microorganism is grown on different substrates, it often produces enzymes having different specificities as well as the same enzymes in different proportions. For example, the extracellular xylanase activities produced by *A. fumigatus* depends on the nature of the lignocellulosic substrate it is grown on (Stewart *et al.*, 1985). These activities were separable into three main groups of proteins by gel-filtration chromatography, the type and amount of each one depended on the growth substrate. The different types and amounts of enzymes produced by *T. reesei* RUTC30 when it was grown on canola meal and other substrates used in this study were compared.

Canola production has increased by over 300 percent in the last decade (Vaisey-Genser, 1987), and consequently there is more canola meal available. The present use of canola meal as animal feed is restricted because it is toxic in large doses. This project investigates an alternative use for canola meal, that is as a substrate for xylanase enzyme production by *T. reesei*. This section gives some background information about canola and in particular canola meal.

Rapeseed oil was first used by ancient civilizations in Asia and Europe as a lamp oil. The need for Canadian production of the oil began during World War II due to the blockage of European and Asian sources. At this time it was used as a lubricant for steam engines in ships. Rapeseed oil was not used for edible purposes until the end of World War II, because it was harmful in large quantities due to high levels of erucic acid. In 1974, Baldur Stefansson a Canadian plant breeder developed the first "double-low" variety with both reduced erucic acid and glucosinolate levels. This type, called Tower from the *Brassica napus* variety became known as "canola". On September 12, 1986, it was ammended by the Trade Marks Branch of Consumer and Corporate Affairs to indicte that canola oil must contain less than two percent erucic acid, and the solid component of the seed must contain less than 30 micromoles per

gram of glucosinolates. The name canola has become a world wide generic term and is now also used to describe *B. napus* and *B. campestris* seeds.

Canola is grown on a commercial scale in the cooler areas of the world such as Canada, China, Northern Europe, and the Indian subcontinent (Butler *et al.*, 1982; Mickle and Co., 1986). An oversupply of wheat in the late 1960's and early 1970's led many Canadian farmers to diversify their production to canola. Canola oil production has increased by over 300% in the last decade (Vaisey-Genser, 1987)

In 1987 a Canadian record for canola production was set, this was 3.852 million tonnes of seed. Over the past 10 years Canada has become the largest exporter of seed in the world, with exports accounting for half of the canola produced in Canada. The Canola Council of Canada plays a central role in the Canadian canola industry by not only providing support to farmers and researchers, but also bringing together everyone with a stake in the industry thereby facilitating decisions which will be of benefit to the industry as a whole (Vaisey-Genser and Harris, 1987).

Canadians are the largest per capita consumers of canola oil in the world. Canola oil accounts for 60% of all edible vegetable oil products manufactured in Canada. It accounts for 80% of the liquid or salad oils, 50% of the shortening, and 40% of the margarines (Vaisey-Genser and Harris, 1987).

In 1985 and 1986 nutritional studies (Mattson and Grundy, 1985) showed that diets high in monounstaurated fatty acids were effective in lowering cholesterol. Canola oil is cholesterol free and is also characterized by a high content of monounsaturated fatty acids, a feature which is now considered a nutritional plus. The composition of canola oil is shown in Table 2.1 (Vaisey-Genser, 1987).

Liquid canola oil remains clear and free-flowing even when refrigerated. Canola oil is an ideal salad oil with a bland flavor light color and delicate aroma. It also makes an excellent frying oil because it is more temperature-light-air-stable than most vegetable oils. Canola oil leaves food 5 to 10% lower in calories than those fried in shortening (Eskin, 1987).

Table 2.1: Composition of Canola Oil (Vaisey-Genser, 1987)

Free fatty acid (as oleic acid) maximum by mass	1.0%
Moisture and impurities combined maximum by mass	0.3%
Chlorophyll, maximum	30 ppm
Neutral oil, minimum by mass	98.5%
Loss, maximum by mass	1.5%
Phosphorus content, maximum	50ppm
Erucic acid, maximum by mass	2.0%

Canola oil is composed of 96–99% triglycerides, the balance of which is a mixture of components which are detrimental to the quality of the finished product. Removal of these components is the main objective of processing while at the same time, triglyceride loss must by minimized. First, the canola seed are passed through operations which remove foreign particles, then canola oil is extracted by rolling or flaking the seed to fracture their coat and rupturing the oil cells. The remaining flakes are then cooled, compressed into large cake fragments, then the fragments are solvent extracted to ensure maximum oil removal. The crude oil is then passed through a degumming process, refined and bleached (Eskin and Bacchus, 1987).

The cake fragments which remain after oil extraction are steam-stripped to remove solvent and then dried. The meal emerges free of solvents, contains 1.5% residual oil, and has a moisture content of 8 to 10%. After cooling, the meal is often granulated to a uniform consistency and is either pelletized or sent directly to storage ready for marketing as a high protein feed supplement for livestock and poultry (Eskin and Bacchus, 1987). Table 2.2 shows the composition of canola meal (Vaisey-Genser, 1987). Some of the products produced from canola seed are shown in Figure 2.2

The present use for canola meal is limited to animal feed, although it has detrimental effects in large doses. For example, canola seed coat has been shown to depress protein

Table 2.2: Composition of Canola Meal (Vaisey-Genser, 1987)

Moisture, maximum by mass	11.0%
Fat, minimum by mass	0.5%
Protein, minimum by mass	35.0%
Fibre, maximum by mass	12.0%
Glucosinolates, maximum	30μm/g
Screen analysis by mass:	
through 1.7mm sieve	90%
through 2.0mm sieve	100%

digestability and reduce bioavailability of Cu and Zn in animal diet (Ward and Reichart, 1986). It has also been shown to cause li er damage in poultry and taint eggs (Butler *et al.*, 1982). The phytic acid present in canola meal is harmful to animals because it affects the availability of minerals (Bell and Keith, 1987).

Canola meal is widely used in Canada in poultry rations because of its nutritional value, and its availabillity at a competitive price relative to other protein supplements (Robblee *et al.*, 1987). Canola meal is also used as a protein supplement in swine rations, aproximately 20% of the canola meal available in Canada is fed to swine (Bell and Aheme, 1987). Canola meal has become internationally accepted as a protein source for cattle (Fisher and Ingalls, 1987), and is also being used as a source of protein for salmon (Fagerlund *et al.*, 1987).

A disadvantage of using most lignocellulosic materials such as canola meal as substrates for enzyme production or saccharification to component carbohydrates is the need for costly pretreatment (Royer and Nakas, 1987). Pretreatment enhances the susceptibility of these materials to enzymatic or microbial attack (Szczodrak, 1988). For example waste sludges have been rendered accessible to enzymatic attack by the pulping process. The type of pulping used for example sodium sulfide or sodium hydroxide

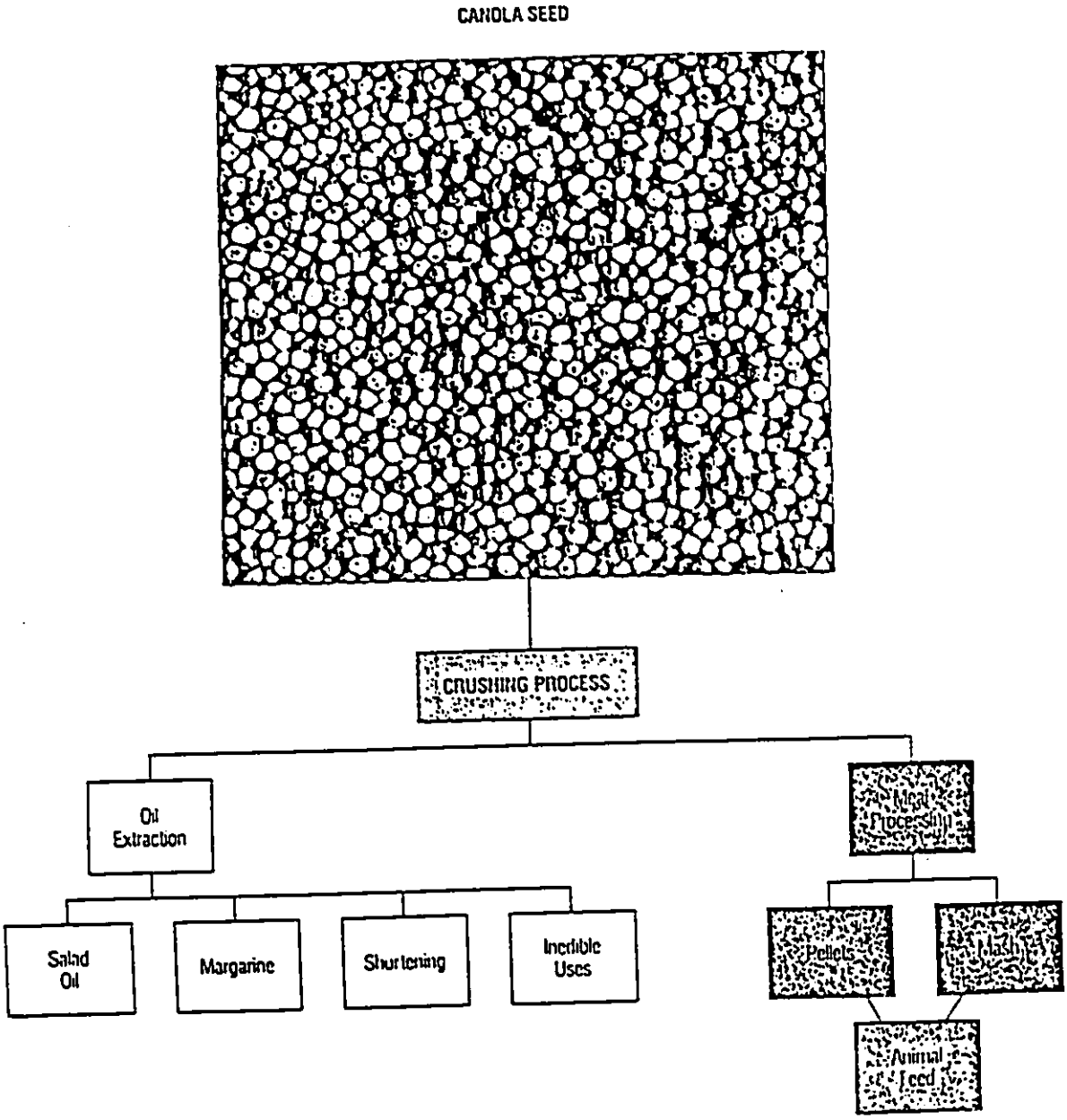

Figure 2.2: Canola Seed Products (Vaisey-Genser, 1987)

to delignify the wood affects the susceptibility to enzymatic (Royer and Nakas, 1987). This investigation will look at the use of heat pretreatment because it is necessary for sterilization.

2.3 The Future of Lignocellulose Conversion

The future of the fermentation biotechnology industry depends to a great extent on the price of its carbohydrate raw materials. Lignocellulose is a potential source of less expensive fermentable sugars provided that (1) all of the components of the heterogeneous lignocellulosic material are used and (2) economical pretreatment methods are developed which increase the yield of glucose and other sugars from biomass hydrolysis (Dale, 1987). Developments in these areas will signify the beginning of a new era in resources bioprocessing.

Chapter 3

Materials and Methods

The experimental methods and the analytical procedures followed are described in detail in this chapter.

3.1 Microorganism

The fungus *Trichoderma reesei* RUT C-30, NRRL 11400 was used for enzyme production in this study. The microorganism was maintained by regular transfer on Potato Dextrose Agar (Difco) slants and kept at 4°C.

3.2 Media

The composition of the media used in this study are outlined in this section.

3.2.1 Medium Used for Enzyme Production

Vogel's medium (Montenecourt *et al.*, 1977) was used for inoculum and enzyme production. The basal medium was composed of: Vogel's salt solution (20 ml), Vogel's vitamin solution (1 ml), Vogel's mineral solution (0.1 ml), Tween 80 (1:9 dilution with

water) (Fisher) (20 ml), and distilled water up to 1 l of medium. The pH of the media was adjusted to 5.5–5.7 using 2N HCl. The Vogel's salt solution was composed of: 125 g Na-citrate·$2H_2O$ (BDH), 250 g K_2HPO_4 (Fisher Scientific), 100 g NH_4NO_3 (Anachemia), 10 g $MgSO_4 \cdot 7H_2O$ (BDH), 3.8 g $CaCl_2 \cdot 2H_2O$ (Anachemia), and 775 ml of distilled water. The Vogel's vitamin solution contained 0.5 mg Biotin (Sigma), 200 mg muoinositol (Sigma), 20 mg Ca-pantothenate (Sigma), 20 mg Pyridoxine HCl (Sigma), 15.5 mg Thiamine (Sigma), and distilled water up to 100 ml. The Vogel's trace minerals solution was composed of: 5 g Citric acid·H_2O (Anachemia), 5 g $ZnSO_4 \cdot 7H_2O$ (Anachemia), 1 g $Fe(NH_4)_2(SO_4)_2 \cdot 5H_2O$ (Baker), 0.25 g $CuSO_4 \cdot 5H_2O$ (Fisher), 0.05 g $MnSO_4 \cdot H_2O$ (Baker), 0.05 g H_3BO_3 (Anachemia), 0.05 g $Na_2MoO_4 \cdot 2H_2O$ (Sigma), and distilled water up to 100 ml.

The canola meal, *Brassica napus cr*, was purchased locally from a feed store. It contained 11% moisture, 41% protein, 8% ash, 4% ether extractables and 36% nitrogen free extract. The nitrogen free extract contained 28% polysaccharides and 8% phenolics (Blair *et al.*, 1987). Solka-floc, a delignified and ball milled pulp preparation was obtained from Brown and company (Berlin, NH, U.S.A.). The other substrates, cellulose, glucose, lactose, sucrose, oat spelt xylan and larchwood xylan were obtained from the Sigma Chemical Company (St. Louis , MO, U.S.A.).

The basal medium was supplemented with 1–8% weight/volume (w/v) of the carbon sources previously described. This medium was also supplemented with bactopeptone (usually 10% of the carbon source, weight/weight (w/w)). In media containing 1–8% canola meal (w/v), 0.2% bactopeptone was added instead of 10% used in the other cases in order to make use of the protein already present in the canola meal. All tests were run in duplicate, some on at least two different occasions. The averaged results were plotted, and any data which varied by more than ±15% were discarded. Unless otherwise specified all substrate concentrations can be assumed to be in terms of w/v.

3.2.2 Effect of Culture Conditions on Xylanase Production

The production of xylanase was monitored using the following variations in culture conditions. The effect of marbles on xylanase enzyme production was tested using 2% canola meal. This test used four flasks, 2 of which each contained 2 marbles, and 2 which did not. The effect of Tween 80 on xylanase production was tested using 2% canola meal. The test was run using four flasks, two contained 0.2% v/v Tween 80, the other 2 contained water in place of Tween 80. The effect of bactopeptone on xylanase production was studied by adding 3.3% and 10% (w/w) bactopeptone relative to carbon source to flasks containg 6% canola meal as substrate. The effect of inoculum volume on xylanase production was tested by adding 2.5%, 5%, and 10% (v/v) to flasks containing medium with 4% canola meal as substrate. The effect of initial pH on xylanase production was studied by adjusting the pH of the media containing 4% canola meal prior to autoclaving. The initial pH values were adjusted to 5.6, 5.0, 4.0, and 3.0. The pH values determined after autoclaving were considered to be the initial pH in these tests. The effect of culture temperature was determined by incubating the flasks containing 4% canola meal at 28°C and 37°C. The effect of sterilization pretreatment on canola meal was determined by comparing enzyme production in flasks containing 4% canola meal which had been sterilized to those which had not.

3.3 Enzyme Production

3.3.1 Preparation of Inoculum and Enzyme Production

The inoculum was prepared by transferring spores from an agar slant to 100 ml Vogel's growth medium supplemented with 1 g glucose and 0.1 g of bactopeptone. The inoculum was grown in 250 ml Erlenmeyer flasks each containing 25 ml of the medium and 2 glass marbles to prevent clumping of the cultures. The preparation was incubated with shaking at 200 rpm for 2 days at 27°C. The inoculum (2–5% v/v) was asceptically

with the growth media for enzyme production. The enzyme production was carried out in 500 ml Erlenmeyer flasks, each containing 200 ml of the desired medium. The media were autoclaved at 121°C for 15 minutes and cooled prior to inoculation. After inoculation, the cultures were incubated with shaking at 200 rpm at 27°C. Xylanase production was followed at regular intervals usually after every 2–3 days. Samples were asceptically withdrawn and the pH was tested. The samples were then centrifuged and the supernatant liquid was filter sterilized using a 0.45 μm Minisart filter. The filtrate was diluted appropriately and then tested for xylanase activity.

3.3.2 Harvesting and Storage of the Enzyme

The entire culture medium was generally harvested for enzyme after xylanase activity reached a maximum value, this was usually after 9–12 days. To harvest the enzyme, the culture was passed through cheese cloth and then centrifuged at 3000 × g for 20 minutes. The supernatant was then centrifuged at 8000 × g for 30 minutes, filter sterilized using a Nalgene 0.45μm filter and stored in presterilized vials at 2°C.

3.4 Additional Enzyme Preparations Used in this Study

The additional enzyme preparations used in this study were Gamanase, Novozym and the *T. reesei* culture dialysed enzyme system.

Gamanase is a hemicellulase preparation capable of randomly hydrolyzing β(1–4) bonds in mannans, galactomannans and glucomannans. Novozym 188 is a multienzyme preparation composed of different hemicellulases which hydrolyse arabans, galactans, xylans and other hemicellulases. Both enzymes were obtained from NOVO Laboratories Ltd., Bioindustrial Group, Lachine, Quebec, Canada. The *T. reesei* dialysed enzyme system was a preparation of enzymes from *T. reesei* RUT-C30 which had been

cultivated in a 25 L fermentor using Solka floc as a substrate (Khan *et al.*, 1989*a*).

The Gamanase and Novozym enzymes were dialysed after appropriate dilution for 24 h at 2°C. To avoid possible damage by these enzymes, the dialysis tubing was changed every 4 h. After dialysis, these enzyme preparations were filter sterilized using a 0.45 μm filter and stored in pre-sterilized vials at 2°C.

3.5 Analytical Methods

In the study of the production of xylanase and some of the other enzymes involved in the conversion of cellulosic and lignocellulosic materials, the reducing sugar concentration, protein and nitrogen content, biomass concentrations and enzyme activity were followed.

3.5.1 Measurement of Reducing Sugar Concentrations

The concentration of reducing sugars glucose and xylose were estimated using the dinitrosalicylic acid (DNS) reagent (Miller, 1959). A blank was run using 0.5 ml water. To 0.5 ml of each sample was added 1.0 ml citrate buffer and 3 ml DNS Reagent. The samples were the filtrate from the culture medium. The citrate buffer was made up of 10.51g of citric acid·$2H_2O$ (Anachemia) in 1 l of distilled water. The pH was adjusted to 4.8 using 5N NaOH. The DNS Reagent is composed of: 13.25g 3,5–Dinitrosalicylic acid (Sigma), 25g NaOH (BDH), 380g Rochelle Salt (NaKTartrate) (BDH), 9.5 ml liquid Phenol (Baker), 10.4g Sodium Meta–Bisulfate (Baker), in 2 l of distilled water.

The 4.5 ml reaction mixtures were boiled 15 min, then cooled to room temperature in a water bath, 16 ml water was added to each sample mixed and the absorbance read at 575 nm. In order to be in the linear range of the test, the absorbance should not be lower than 0.4 or higher than 1.8.

To make the standard curve shown in Appendix A, Figure A.1, a 1% sugar solution

of glucose or xylose was diluted to concentrations from 1–5 mg/0.5 ml using water. The amount of glucose or xylose in unknown samples was determined by comparing the optical density to that found on the standard curve.

3.5.2 Determination of Sugar Composition Using High Performance Liquid Chromatography

Sugar composition of the polymer produced as a result of hydrolysis of canola meal was determined by high performance liquid chromatography (HPLC) using a Polypore PB column (4.6×22 mm) (Brownlee Labs, Santa Clare, CA, U.S.A.) and an infra red refractive index detector. The column was kept at 80°C and deionized water was used as the mobile phase at a flow rate of 0.3 ml/minute for 30 minutes. The injection volume was 20 μl and the attenuation range was 128.

3.5.3 Determination of Protein and Nitrogen Content

Protein and nitrogen content was determined using the Folin-Phenol Reagent (Lowry *et al.*, 1951). The reagent is composed of two solutions, fifty ml of the solution I which contains 1 ml of 1% $CuSO_4 \cdot 5H_2O$ (Fisher), 1 ml of 2% Sodium Citrate (BDH), and 48 ml of 5% Na_2CO_3 (BDH). This mixture must be prepared fresh before use. The solution II contains 2N Phenol; Folin-Ciocalteau (Fisher), diluted 1:1 with water. A blank was run using 0.5 ml of water. The 0.5 ml test samples were combined with 0.5 ml 1 N NaOH and heated in a boiling water bath for 5 min. The test samples were the filtrate from the culture medium. After cooling, 2.5 ml Reagent I was added, mixed, and let stand at room temperature for 10 min. Then 0.5 ml Reagent II was added, mixed and let stand for 30 min. The absorbance was read at 600 nm. In order to be in the linear range of the test, the absorbance values used must not be less than 0.3 or greater than 1.0.

To make the standard curve (Appendix A, Figure A.2), 1 ml of albumin stock

protein solution (Sigma Chemical Co. No. 905–10, 100 g/l total protein by biuret) was diluted to 100 ml in water. Appropriate dilutions were then made to produce values ranging from 25–500 μg protein per 0.5 ml test volume. The amount of protein in unknown samples was determined by comparing the optical density to that on the standard curve.

3.5.4 pH Measurement

The pH measurements were conducted using a radiometer pHM82 Standard pH meter with a combination electrode. The instrument was calibrated using pH reference buffer solutions of 4.01 and 7.00 (Canlab) prior to each measurement.

3.5.5 Biomass Measurement

The extent of cell growth was monitered by measuring the dry weight of the cells. For each measurement a 5 ml sample from the culture was centrifuged at 5000 × g for 20 minutes. The pellet was washed with distilled water using suction filtration. The residue on pre-weighed Whatman #4 filterpaper was transferred to a pre-weighed aluminum dish. These were then dried at a temperature of 105°C to a constant weight. Biomass determinations were performed when 1% glucose, 1% lactose, and 1% sucrose were used as substrates.

3.6 Enzyme Assays

3.6.1 Xylanase Activity Determination

Xylanase activity was determined using oat spelt xylan as substrate (Khan *et al.*, 1986). The assay was carried out by mixing 0.5 ml of an appropriate dilution of enzyme preparation with 1 ml of 1% solution of oat spelts xylan in 1 ml of citrate

buffer. The blank contained 0.5 ml of water and 1.0 ml of buffer, the enzyme control contained 0.5 ml enzyme and 1.0 ml buffer, and the substrate control contained 0.5 ml water and 1.0 ml substrate.

The mixtures were incubated for 30 minutes at 50°C immediately after addition of the substrate. Three ml of DNS reagent was added to stop the reaction at the end of the incubation. Then continue with the DNS method for sugar estimation (Section 3.5.1).

Enzyme dilutions were carried out by continually diluting a 0.5 ml sample from the enzyme preparation in half (1/2, 1/4, 1/8, up to 1/256). According to Khan *et al.* (1986) enzyme activity depends on enzyme dilution and the substrate used. Appendix B shows the nonlinear relationship between enzyme activity versus enzyme concentration. For the calculation of activity, a dilution of enzyme was used which would release 2 mg of sugar in 30 minutes. This range was selected because the absorbance measurements for sugars using the DNS method are most reproducible in the 1–3 mg/test range. The error size due to leveling off of the curves are also smallest in this region because 2 mg is located in a relatively linear region.

Activity is expressed in the international units (IU) which are defined as the amount (or volume) of enzyme required to liberate 1 μmol of sugar per minute and is calculated under the described assay conditions using Equation 3.1.

$$Activity(IU/ml) = \frac{mg\ sugar/test \times 1000(\mu g/mg) \times 2 \times dilution}{150(MW\ of\ xylose) \times 30(min)} \qquad (3.1)$$

Method for Determination of pH Optimum for the Xylanase Enzyme System

The determination of the pH optima of the xylanase enzyme systems produced on Solka-floc and canola meal was done as outlined previously for the xylanase activity determination, except the pH of the buffer was varied using citrate-phosphate and phosphate buffer systems (Gomori, 1955). It was necessary to use this combination

Table 3.1: Citrate-Phosphate and Phosphate Buffer Preparation for Determination of pH Optimum for the Xylanase Enzyme System

pH	Buffer	A	B	C	D
		Volume(ml)			
3.0	Citrate-Phosphate	39.8	10.2	–	–
4.0	Citrate-Phosphate	30.7	19.3	–	–
5.0	Citrate-Phosphate	24.3	25.7	–	–
6.0	Citrate-Phosphate	17.9	32.1	–	–
6.0	Phosphate	–	–	87.7	12.3
7.0	Phosphate	–	–	39.0	61.0
8.0	Phosphate	–	–	5.3	94.7

A: 0.1M Citric Acid (19.21 g in 1000 ml)
B and D: Dibasic Sodium Phospate (53.65 g $Na_2HPO_4 \cdot 7H_2O$ in 1000 ml)
C: Monobasic Sodium Phosphate (27.8 g in 1000 ml)

to ensure that the buffering capacity of the buffers was within the range of the pH values tested. The buffers were prepared using the amounts shown in Table 3.1 The volumes given in the table are approximate and may not give the desired pH values. The solutions A and B or C and D were mixed and then diluted to a total of 200 ml with distilled water.

The additions of 1% xylan to each of these buffers did not change the pH by more than ± 0.05. The difference between the enzyme activities determined using the citrate phosphate and phosphate buffers at pH 7.0 were less than 5% and the points determined

for the citrate phophate buffer were plotted at pH 6.0 (Figure 4.14).

Method for Determination of Temperature Optimum for the Xylanase Enzyme System

The determination of the temperature optima of the xylanase enzyme systems produced on Solka-floc and canola meal was done as outlined previously for the xylanase activity determination, except the incubations were run at 20, 30, 40, 50, 55, 60, and 70°C using a temperature controlled water bath. The incubations for this test were done without shaking, but the test tubes were shaken manually every ten minutes.

Effect of Temperature on the Stability of Canola Meal and Solka-floc Enzyme Systems

The stability of the enzyme systems produced on canola meal and Solka floc were determined at 37°C and 50°C by keeping samples of these enzyme systems at each of these two temperatures. The activity of each was tested every 2–3 days in the case of the samples at 37°C, and over a period of 24 hours for the samples at 50°C.

The stability of the enzyme systems at 2°C was tested by determining the activity of the oldest samples available at the time of the test, this was approximately 1 year.

Effect of Dialysis on Enzymatic Activity

Five ml samples of enzyme obtained using canola meal and Solka floc as substrates were dialysed against water. Dialysis was carried out over a period of approximately 10 hours with three 2 l changes of water. The volume before and after dialysis was measured as well as xylanase activity and protein content before and after dialysis.

Inhibition and Stimulation Tests

The effect of metal ions, thiol groups, reducing agents and chelating compounds were used to study inhibition and stimulation of the enzyme system produced using canola meal as substrate. Thirty mM solution of each of the compounds listed in Table 3.2 were tested.

The enzymes were assayed exactly as described above except the reaction mixtures shown in Table 3.3 were used. The change in activity was determined relative to the control.

3.6.2 Cellulase Activity Using Filter Paper as Substrate

This assay is the same as the xylanase assay just described in subsection 3.6.1 except for two main differences (Mandels *et al.*, 1976; Khan *et al.*, 1986). First the substrate is a 1 × 5 cm strip of Whatman #4 filter paper which must be vortexed to ensure it is completely immersed in the incubation mixture. Second the incubation period is 60 minutes (as opposed to 30).

Enzyme activity was determined using a dilution of enzyme which will release 2 mg of sugar in 60 minutes as shown in Equation 3.2

$$Activity(IU/ml) = \frac{mg\ sugar/test \times 1000(\mu g/mg) \times 2 \times dilution}{180(MW\ of\ glucose) \times 60(min)} \qquad (3.2)$$

3.6.3 β-Xylosidase Activity Determination

β-xylosidase activity was determined using p-nitro-β-d-xylopyranoside (NPX) as a substrate (Khan *et al.*, 1986). When the NPX is cleaved by β-xylosidase, xylose and p-nitrophenol are released. A solution of p nitrophenol mixed with Na_2CO_3 is yellow in color and can be measured spectrophotometrically.

A 1 M sodium carbonate solution was prepared by dissolving 10.6 g Na_2CO_3 into 100 ml of water. In order to carry out the enzyme assay, the test samples contained

Table 3.2: Metal ions, reducing agents and chelating compounds tested for their effect on the dialysed xylanase preparation produced using canola meal as a carbon source

Compound Tested	Concentration (mM)
Metal Ions	
KCN (K^+)	10
$HgCl_2$ (Hg^{2+})	10
$AgNO_3$ (Ag^+)	1
$ZnCl_2$ (Zn^{2+})	1
$MnSO_4$ (Mn^{2+})	10
$CoCl_2$ (Co^{2+})	10
$CaCl_2$ (Ca^{2+})	10
$MgCl_2$ (Mg^{2+})	10
Thiol and Reducing Agents	
Glutathione	10
Cysteine	10
Dithiothreitol	10
Ascorbic Acid	10
Chelating Compounds (Samples Undialysed)	
Ethylenediaminetetraacetic Acid	10
Sodium Azide	10
Nitrilotriacetic Acid	10
N-ethylemaleimide	10

Table 3.3: Volumes of components added to test the effect of various compounds on xylanase enzyme activity

Sample	Substrate	Buffer	Inhibitor	Enzyme
		Volume (ml)		
Blank	–	1.5	–	–
Substrate Control	0.5	1.0	–	–
Enzyme Control	–	1.0	–	0.5
Control	0.5	0.5	–	0.5
10 mM Control	0.5	0.5	0.5	–
10 mM Substrate	0.5	–	0.5	0.5
1 mM Control	0.5	0.95	0.05	–
1 mM Substrate	0.5	0.45	0.05	0.5

1 ml NPX substrate plus 1 ml of appropriately diluted enzyme solution. The blank contained 1 ml of water plus 2 ml citrate buffer, and the substrate control contained 1 ml water plus 1 ml of substrate.

Each sample was incubated for 15 min at 50°C. Then 2 ml of the Na_2CO_3 mixture was added to each sample and the absorbance was read at 400 nm. Prior to performing the assay, a standard curve must be prepared. The standard was prepared using a 4 mM p-nitrophenol stock solution (0.139 g p-nitrophenol and 250 ml distilled water) diluted 1:10 with citrate buffer to obtain a 0.4 mM concentration.

The standard curve (Figure A.3, Appendix A) was prepared by adding 0.1 to 1.0 ml of the p-nitrophenol solution to citrate buffer to make up to a total of 1 ml (0.04 to 0.4 mM in concentration). The 1.0 ml of water and 2.0 ml of Na_2CO_3 solution were added and the absorbances were read at 400 nm. Using the slope of the curve the concentration in the test solution is equal to absorbance multiplied by 0.20 mM.

The problem of nonlinearity of enzyme activity with enzyme dilution is again observed (Khan *et al.*, 1986). Xylosidase units per ml were defined as the inverse of the enzyme dilution that gives an absorbance between 0.8 and 1.2 using a 0.1% 4NPX solution as substrate during a 15 min assay period. The calculation of activity is shown in equation 3.3.

$$Activity(IU/ml) = \frac{mM\ \ NPX/test \times 1000(\mu g/mg) \times dilution}{15(min)} \qquad (3.3)$$

3.6.4 Acetyl-Xylan Esterase Activity

Acetyl-xylan esterase activity was measured according to Khan *et al.*, 1989*b*. The substrate used was a 2% (w/v) solution of natural xylan in 0.4M phosphate buffer pH 6.5. Half a milliliter of an appropriate dilution of the enzyme was added to 0.5 ml of this substrate. The blank contained 0.5 ml phosphate buffer plus 0.5 ml water, the enzyme control contained 0.5 ml substrate plus 0.5 ml water. The mixtures were

incubated for 30 min at 37°C. The reaction was stopped by adding 1 ml of 0.12M H_2SO_4 containing an internal standard for the gas chromotograph analysis. The internal standard solution contained 100 ml isobutyric acid solution (20 g/l), 40 ml formic acid (distilled) and 12 ml 10 N sulfuric acid per litre of distilled water. The samples were then centrifuged using a desk top centrifuge for 3 minutes, and kept frozen till analyzed. One milliliter of each sample was transferred to gas chromatographic vials for gas chromatographic analysis to determine the amount of acetic acid (mg/l) which had been released. These calculations were based on the amount of isobutyric acid present in the internal standard and were read from the gas chromatographic tracings. Along with appropriate controls, a series of 0 to 10 times dilution of the enzyme was run. The dilution after which the reaction rate became linear was used for the calculation of enzyme units. One international unit (IU) of acetyl-xylan esterase was defined as the amount of enzyme needed to liberate 1 μmol of acetic acid from 10 mg of substrate in 1 minute. The activity was calculated as shown in equation 3.4.

$$Activity(IU/ml) = \frac{\mu g/ml\ of\ acid/test \times 2 \times dilution}{60(MW\ of\ acetate) \times 30(min)} \tag{3.4}$$

3.6.5 Additional Enzyme Assays

The enzyme preparations from *T. reesei*, Gamanase and Novozym were assayed for cellulase, and various glucanase and hemicellulase activities using the appropriate dilutions. The substrates used for measuring these activities are shown in Table 3.4. For the estimation of hemicellulase acitivites, substrates such as arabinogalactan, galactan, and galactomannan respectively were prepared from larch wood, gum arabic and gum locust beans, (Sigma Chemical Company). The rest of the substrates were also from the Sigma. Arabinoglucan was obtained by autoclaving a 10–15% canola meal suspension in water then it was centrifuged and filter sterilized. A 1% solution or suspension of these substrates in 0.05M citrate NaOH buffer pH 4.8 was used for enzyme assays.

Table 3.4: Substrates Used for Measuring Various Enzyme Activities

Enzyme activity	Substrate used
Arabinosidase	4NP' Arabinopyranoside
Cellulase	Filter paper
Galactosidase	4NP' Galactopyranoside
1:3α Glucanase	Starch
1:3β Glucanase	Laminarin
1:4β Glucanase	Carboxymethyl Cellulose
β Glucanase	β-Glucan (from barley)
Glucosidase	4NP' Glucopyranoside
Hemicellulase	Arabinogalactan
Hemicellulase	Arabinoglucan (Canola meal)
Hemicellulase	Galactan
Hemicellulase	Galactomannan (Locust beans)
Mannosidase	4NP' Mannopyranside
β-Xylanase	Xylan (Oats spelts)
Xylosidase	4NP' Xylopyranoside

Arabinosidase, galactosidase, glucosidase, mannosidase and xylosidase activities were assayed as outlined earlier for xylosidase for an incubation time of 15 min at 50°C. Cellulase, β- glucanase, and the hemicellulase activities (except arabinoglucan) were assayed as outlined earlier for cellulase and xylanase with an incubation period of 30 min at 50°C. In all the above cases appropriate substrates for the enzymes were used. The rest of the activities listed were assayed over a period of 1 h at 50°C. The hemicellulase activities, arabinogalactan, arabinoglucan, galactan and galactomannan, were calculated as xylan equivalents released using a molecular weight of 150. Specific activity has been expressed as activity/mg of protein.

3.7 Enzymatic Hydrolysis Using the Xylanase Enzyme System

3.7.1 Hydrolysis of Various Agricultural Substrates

The substrates tested for hydrolysis by the xylanase enzyme system include canola meal, corn cobs, corn and wheat brans, larchwood xylan and straw. The corn and wheat brans were pretreated to remove starch (Mares and Stone, 1973). The pretreated material analyzed according to Mares and Stone (1973) and Dubois *et al.* (1956) was shown to contain 48% and 70% polysaccharides respectively. The corn cobs were obtained from Andersons Bed-O-Cob, and the straw was obtained from a local farm.

Saccharification of canola meal and the previously mentioned agricultural substrates was carried out in 60 ml serum vials under aseptic conditions to avoid microbial contamination. Each vial contained 100 or 200 mg of substrate, and an appropriate volume of citrate-NaOH buffer (0.05M, pH 4.8). The vials were sealed with butyl rubber stoppers and pretreated by autoclaving at 121°C for 15 minutes. After cooling, the enzyme preparation under examination was added in the desired amounts and total volume was made to 5 or 10 ml (for 100 or 200 mg of substrate respectively) with deionized

sterilized water. Unless otherwise specified tests for canola meal were always run using an excess of enzyme. All tests were run in duplicate on at least two different occasions. Enzyme and substrate controls were also run in a similar way. All vials were incubated with shaking at 50°C for the designated period of time. After incubation, the contents of each vial was centrifuged at 3000 × g for 20 min and the sugars were estimated in the supernatant before and after acid hydrolysis. Acid hydrolysis was carried out by adding two drops of 0.4 N HCl to the supernatant and heated in a boiling water bath for 15 min. The mixture was neutralized with two drops NaOH after cooling.

In order to study the kinetics of hydrolysis of larchwood xylan, corn bran and canola meal tests were performed using eight serum vials for each of the canola meal and Solka-floc enzyme systems. The tests were run over a period of 24 hours and the contents were analysed for sugars at the times designated.

The minimum amount of enzyme needed to acheive maximal saccharification was determined by incubating up to 20 ml of enzyme preparation from substrate canola meal or Solka-floc enzyme preparations per gram of canola meal. This was performed as outlined earlier over a period of 24 hours. The effect of temperature on the enzymatic hydrolysis of canola meal was determined by running the tests at both 50°C and 37°C. The effect of sterilization pretreatment on canola meal was determined be comparing enzyme production in the case where autoclaving was used compared to when it was not. In the non-autoclaved case, neither vials, canola meal or the buffer mixtures were autoclaved. These tests was run over a period of 24 hours.

3.7.2 A Study of the Enzymes Involved in the Enzymatic Saccharification of Canola Meal

The following subsection describes the procedures used to study the enzymes involved in the enzymatic saccharification of canola meal. The enzyme preparations from *T. reesei*, Gamanase and Novozym were incubated at 50°C for 24 hours. The percent loss

in activity was determined by comparing the loss in enzymatic activity before and after incubation. The hydrolysis of canola meal was carried out as described earlier except the enzyme preparations used were those before and after pretreatment as outlined in the subsection above.

Chapter 4

Results and Discussion

4.1 Production of Xylanase

Enzyme production is dependent on many variables. In order to optimize production of an enzyme, factors such as type and concentration of carbon source, and culture conditions must be studied (Linko *et al.*, 1982). The following section summarizes the effect of various substrates and culture conditions on xylanase production.

The experiments were run in shake flasks using liquid culture. Liquid state was chosen because the majority of *T. reesei* work has been succesfully done using liquid culture. The temperature for enzyme production was chosen to be 28°C because *T. reesei* grows best at this temperature (Dekker, 1983). Vogel's media was chosen because of the proven success of this media for *T. reesei* growth (Montencourt *et al.*, 1977).

4.1.1 Effect of Various Substrates on Xylanase Production

Initially a survey of some of the more expensive substrates which can be used to produce xylanase was carried out. These tests were done to find which of these substrates was the best for xylanase production and to use this substrate for later comparison with the results obtained using canola meal.

Cellulose and Solka-floc as Substrates

Figure 4.1 shows the formation of xylanase in a medium containing 2% cellulose or 2% Solka-floc as a carbon and energy source. Two percent cellulose produced a maximum xylanase activity of 12 IU/ml and 2% Solka-floc gave a maximum xylanase activity of 145 IU/ml, both after 10 days. Marbles were used in this experiment, resulting in slightly lower xylanase activities than in the other experiments which did not use marbles.

In both cases the initial pH was approximately 5.9 and 5.6 for 2% cellulose and 2% Solka-floc media respectively; in the cellulose media the pH rose to approximately 7.2 by the fifth day then it began to drop till it reached 4.0 by the twelfth day. For Solka-floc, the pH dropped down to 4.0 on day 6 and then rose up to approximately 6.1 where it reached a plateau on day 10.

The above results show that for the described culture conditions 2% Solka-floc is a better substrate for xylanase formation than 2% cellulose. The higher activity acheived on Solka-floc rather than cellulose is most likely because Solka-floc contains polysaccharides other than cellulose, such as trace amounts of xylan, which may act as inducers (Bailey and Poutanen, 1989).

Glucose, Lactose or Sucrose as Substrates

Glucose, lactose and sucrose were studied as substrates for xylanase production (Figure 4.2). One percent glucose resulted in xylanase production to the greatest extent with a maximum of 35 IU/ml of activity achieved after 11 days. One percent lactose and sucrose were very poor substrates for xylanase production giving maximum xylanase activities of 8 and 5 IU/ml occuring after 13 days.

The initial pH of the medium with glucose was 5.6 after autoclaving, then it dropped down to 4.1 after 3 days after which it reached plateau at approximately 6.8 after 9 days. The pH was essentially the same for lactose and sucrose, except it reached a

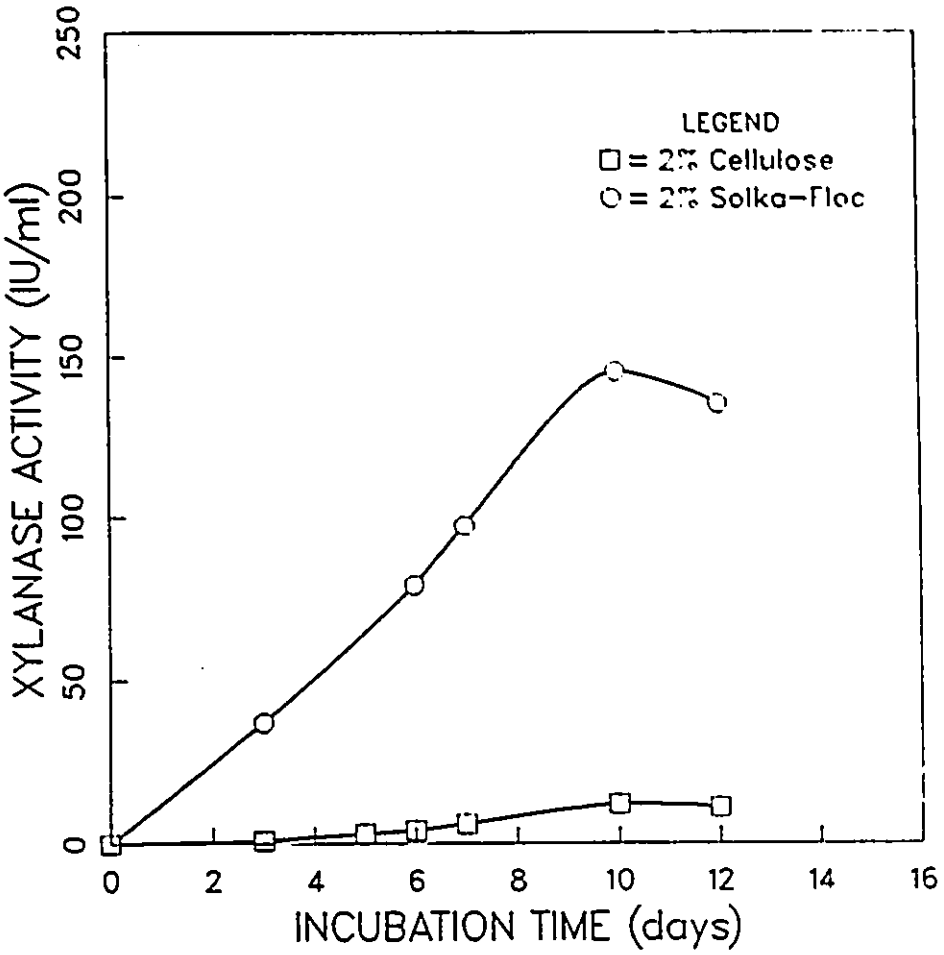

Figure 4.1: Production of xylanase in medium with cellulose or Solka-floc (marbles present)

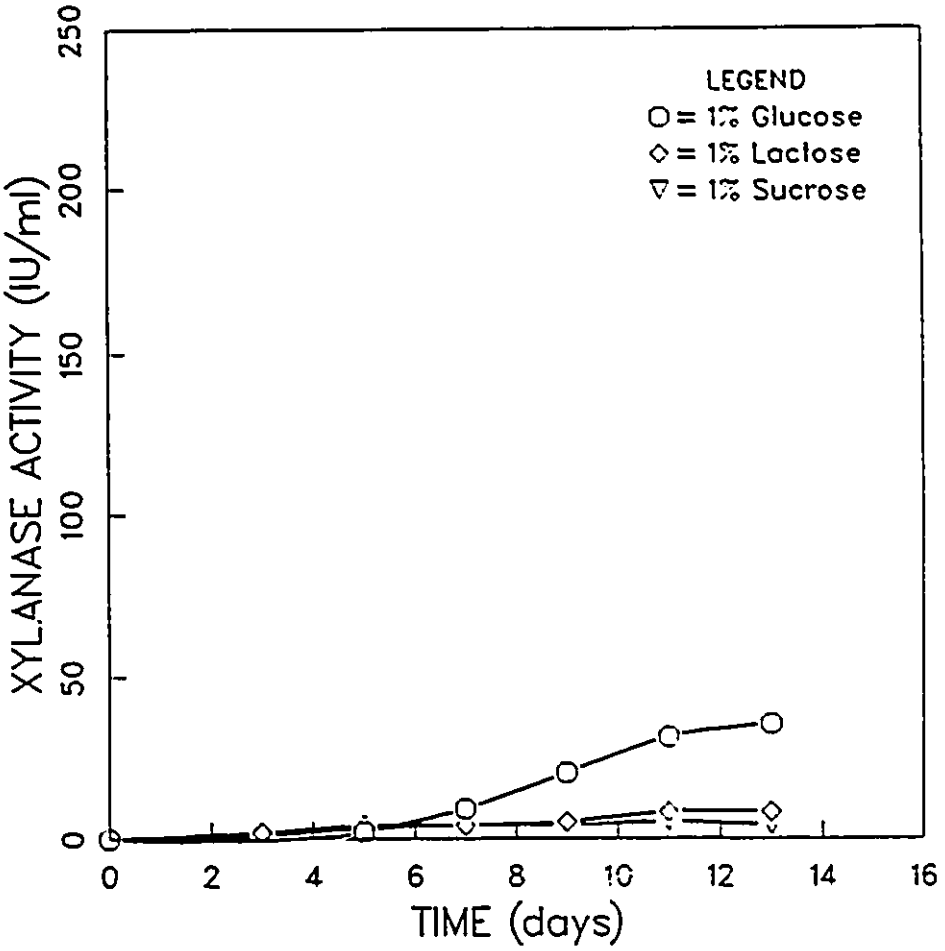

Figure 4.2: Production of xylanase in media containing glucose, lactose, or sucrose

plateau at approximately 7.5 after day 5.

Of the three sugars tested, namely glucose, lactose and sucrose at the 1% level, glucose produced xylanase to the greatest extent, but in very small amounts. Similarly, in medium containing 1% glucose, *T. reesei* QM 9414 has also been shown to produce little or no xylanase activity (Chaudhary and Tauro, 1986). A comparison of results shown in Figures 4.1 and 4.2 indicate that the three sugars tested gave extremely poor yields of xylanase relative to that of Solka-floc.

Oat Spelts Xylan, Larchwood Xylan or Solka-floc as Substrates

The production of xylanase in media containing different types of xylans and Solka-floc is shown in Figure 4.3. One percent larchwood xylan appears to be a slightly better substrate for xylanase production than 1% oat spelts xylan, with maximum activities of 126 and 114 IU/ml respectively acheived on day 9. The maximum xylanase activity of 207 IU/ml was also achieved on day 9 for 1% Solka-floc (Figure 4.3).

The pH of the xylan and Solka-floc containing media were about 5.5 after autoclaving and leveled off on day 5 at pH values of 6.5–6.8. This phenomenon of rise in pH has been observed during the growth of *T. reesei* QM 9414 on hemicellulosic xylans when pH was not controlled (Dekker, 1983). The pH of the medium rose from an initial value of 5.3 to one greater than 6.5. It is not known why the pH of the extracellular medium rises when *T. reesei* is grown on hemicellulose as opposed to other substrates such as cellulose where the pH drops.

Comparison of the results for 1% larchwood xylan with those of Chaudhary and Tauro (1986), show that they obtained a xylanase activity of 9.2 IU/ml after 5 days using *T. reesei* QM 9414, this is much lower than 75 IU/ml (Figure 4.3) reported here for the same period of time. Other workers had also activities as high as 130 IU/ml using 1% larchwood xylan (Robison, 1984), using different experimental conditions such as; a fermenter, pH and oxygen control. Research by Saddler *et al.* (1985) showed

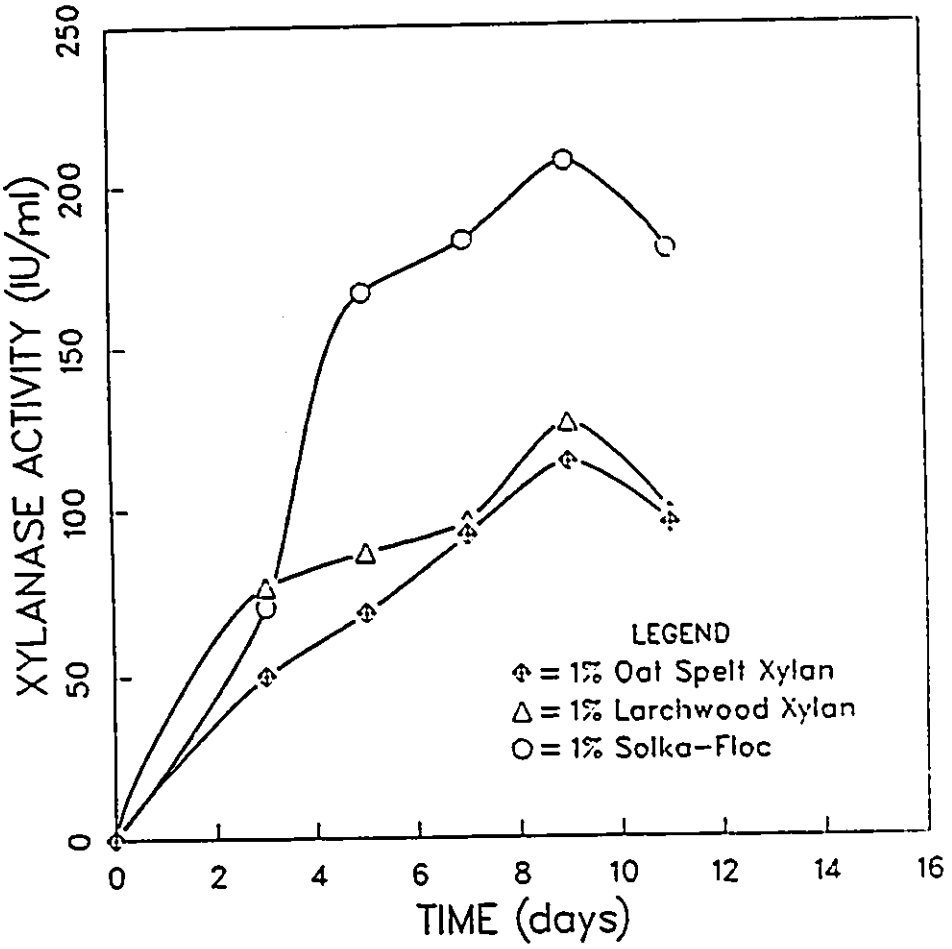

Figure 4.3: Production of xylanase in media containing oat spelts xylan, larchwood xylan or Solka-floc

that xylanase activities of 230 and 30 IU/ml were obtained using 1% larchwood xylan and 1% Solka-floc respectively using *T. reesei* RUT C30. Although the results for 1% larchwood xylan are much higher than the activity of 126 IU/ml shown in Figure 4.3, they are much lower for 1% Solka-floc where an activity of 207 IU/ml was obtained (Figure 4.3). The results for 1% Solka-floc are also higher than those of Tangnu *et al.*, (1981), who obtained a xylanase activity of 114 IU/ml for 1% Solka-floc, with controlled pH and aeration in a fermentor. These differences are probably due to culture conditions such as aeration, pH control, composition of the medium, xylanase assay method used or the use of different strains of *T. reesei*. Evidence for the difference in xylanase activities between various species is also shown by Saddler *et al.* (1985) where xylanase activities of 434 and 240 IU/ml were obtained using 1% Solka-floc and larchwood xylan by *T. harzianum* E58.

Of all the substrates tested in this study, 1% Solka-floc resulted in the highest xylanase activity of 207 IU/ml. For this reason, Solka-floc was selected as a carbon source for subsequent studies.

Biomass production was also followed during the production of xylanase in the media with glucose, lactose and sucrose. The effect of these sugars on biomass production is shown in Figure 4.4. In all cases the biomass levels were the highest on day 3. Of the three sugars tested 1% glucose is the best substrate for the enhancement of biomass production; 63 mg/ml of biomass was produced with this sugar compared to 12 and 13 mg/ml produced using 1% lactose and 1% sucrose respectively.

A relatively high cell concentration is needed to obtain high enzyme activites in media. This is best acheived if there is a relatively rapid initial growth of the microbe. Therefore, during the initial stage of the process, environmental conditions should favor growth of the organism, and then later conditions should favor formation of the enzyme which is a secondary metabolite (Linko, 1982).

When insoluble substrates such as cellulose, Solka-floc and canola meal are used as a carbon source, biomass determination is difficult because it is not possible to separate

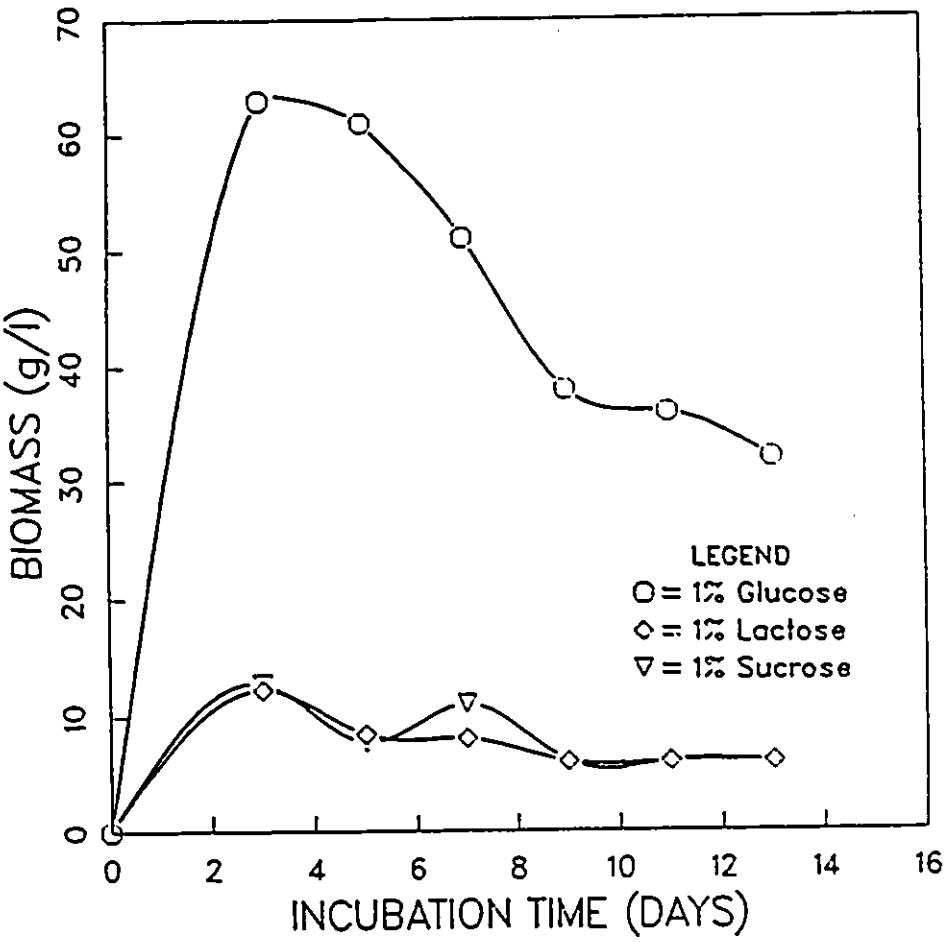

Figure 4.4: Biomass concentrations in media with glucose, sucrose and lactose

the biomass from the insoluble components in these substrates. Therefore since biomass data for these substrates were not available, it was not possible to monitor relationships between microbial growth and enzyme production.

Bactopeptone as a Substrate

The effect of concentration of bactopeptone on xylanse production is shown in figure 4.5. The test compared 3.3% and 10% (w/w) concentrations of bactopeptone relative to a 6% canola meal carbon source. The results show that a maximum activity of 183 IU/ml was obtained using 10% bactopeptone compared to 155 IU/ml acheived using 3.3% bactopeptone. In tests where bactopeptone was not used, a maximum activity of 84 IU/ml was achieved.

An organic nitrogen source is necessary for enzyme formation by *T. reesei*. Although peptone is a good nitrogen source it is not feasible economically on an industrial scale (Linko, 1982). The results show that one advantage of using canola meal as a carbon source is that the addition of an exogeneous nitrogen source is not necessay for enzyme production although addition does enhance it. In order to determine optimal conditions for an industrial process, the use of bactopeptone versus other cheaper nitrogen sources or using none at all would have to be investigated.

4.1.2 Effect of Concentration of Solka-floc on the Production of Xylanase

The results from the survey of the various substrates showed that maximum xylanase production of 207 IU/ml occurred using 1% Solka-floc compared to similar concentrations of other substrates. The purpose of this subsection is to determine the effect of Solka-floc concentration on xylanse formation.

Results reported in Figure 4.6 show that maximum xylanase production was obtained using 1% Solka-floc (Gattinger *et al.*, 1990*a*). The values used in this figure

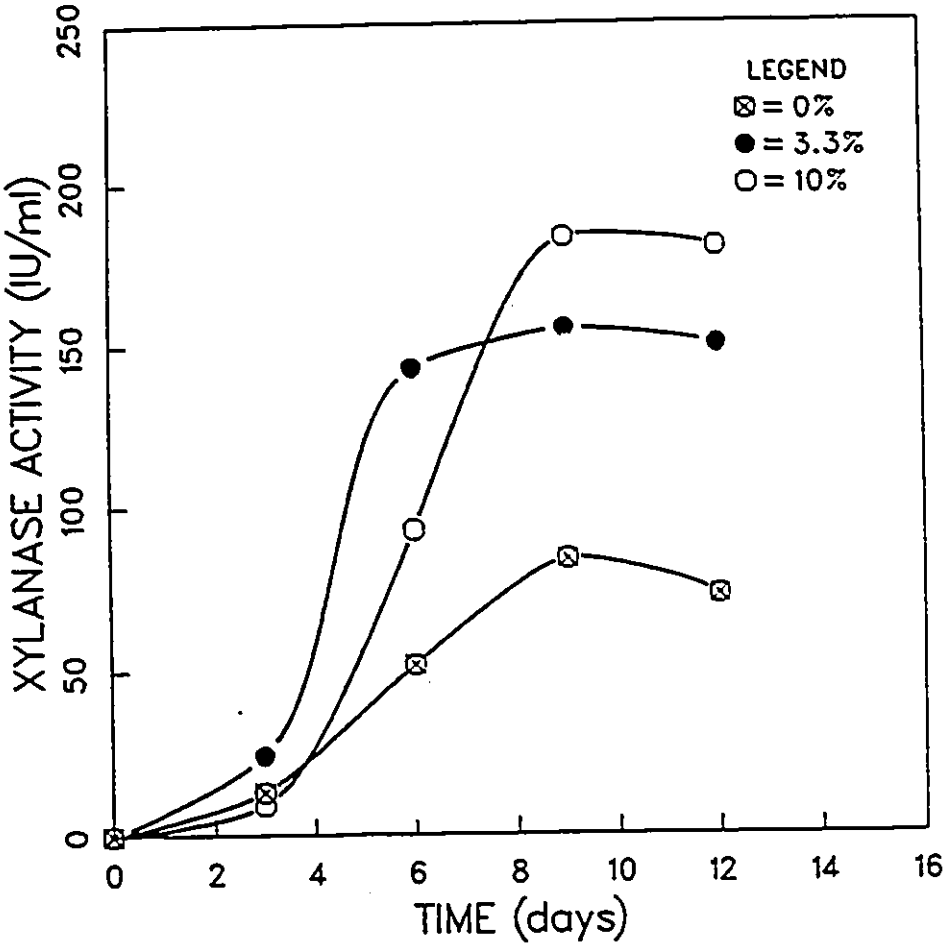

Figure 4.5: Effect of bactopeptone concentration (% of carbon source) on xylanase production in a 6% (w/v) canola meal medium

are the averages of several runs. These results agree with those for larchwood xylan, where maximum xylanase activity was also obtained using 1% larchwood xylan and higher concentrations of the substrate caused partial inhibition of enzyme biosynthesis (Chaudhary and Tauro 1986).

Figure 4.7 shows the changes in pH with respect to the kinetics of xylanase production for 1% Solka-floc. Although the data is not shown, the same profile of pH change was noticed in the 0.5% Solka-floc medium. Whereas for Solka-floc concentrations between 1.5 and 5% the pH rose slightly from 5.5 to 6.3–6.8, then dipped down to approximately 4.0-5.0 between days 5 to 8. This valley was not as greatly pronounced for 3 and 4% Solka-floc.

The concentration of 1% Solka-floc will be used as a basis of comparison for the results obtained using canola meal as a substrate for xylanase production.

4.1.3 Effect of Concentration of Canola Meal on Xylanase Production

The effect of concentration of canola meal on xylanase production is also shown in Figure 4.6 (Gattinger *et al.*, 1990*a*). The figure shows that xylanase activity increased linearly with canola meal concentration. The maximum xylanase activities for each of the percentages of canola meal shown in this figure were obtained between days 7–12. Media with canola meal concentrations above 8% were not tested due to high viscosity of such suspensions which caused difficulties in agitation during enzyme production and difficulties in filtration of samples for enzyme recovery and activity tests.

Figure 4.8 shows the kinetics of production of xylanase using an 8% canola meal medium. The maximum xylanase concentration of 210 IU/ml was achieved on day 12 in this process.

In the first two days, the pH increased from a value of 5.8 to approximately 7.5 and remained there till harvesting. This characteristic pH curve was essentially the

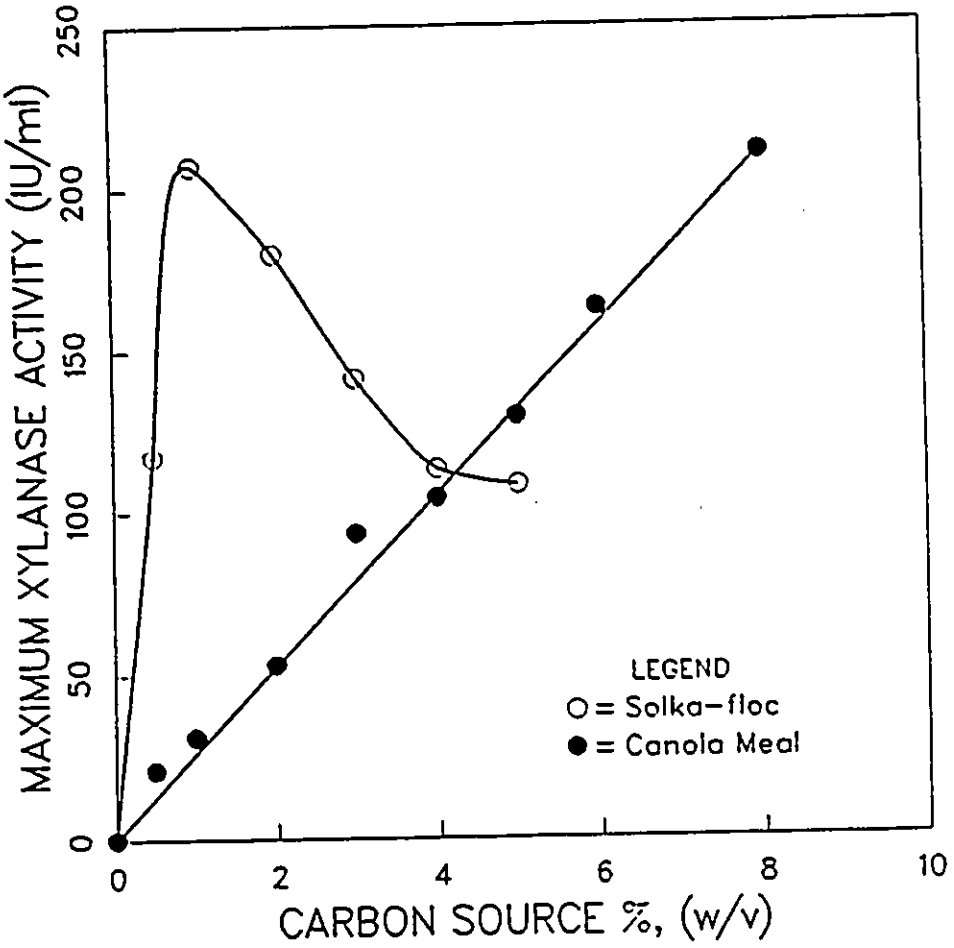

Figure 4.6: Effect of Solka-floc and canola meal concentration on xylanase production

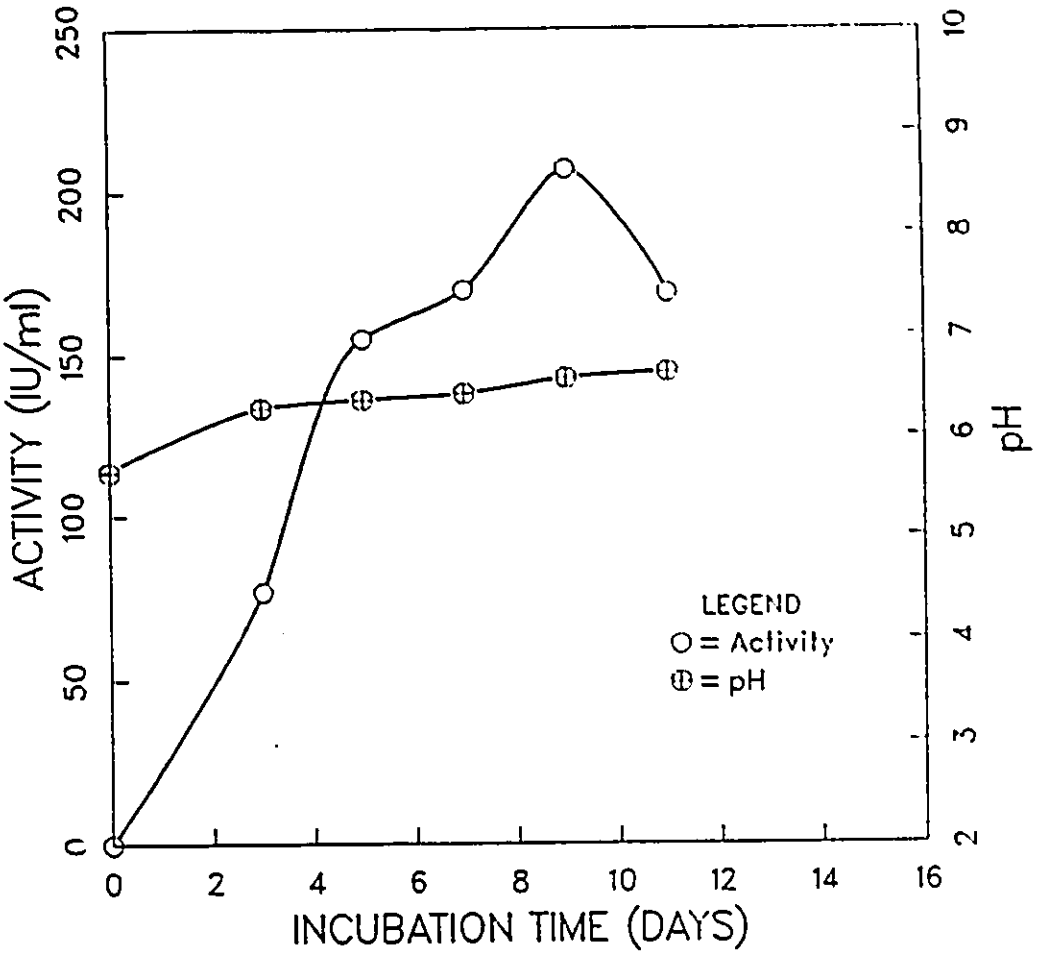

Figure 4.7: Kinetics of xylanase production and pH changes in medium containing 1% (w/v) Solka-floc

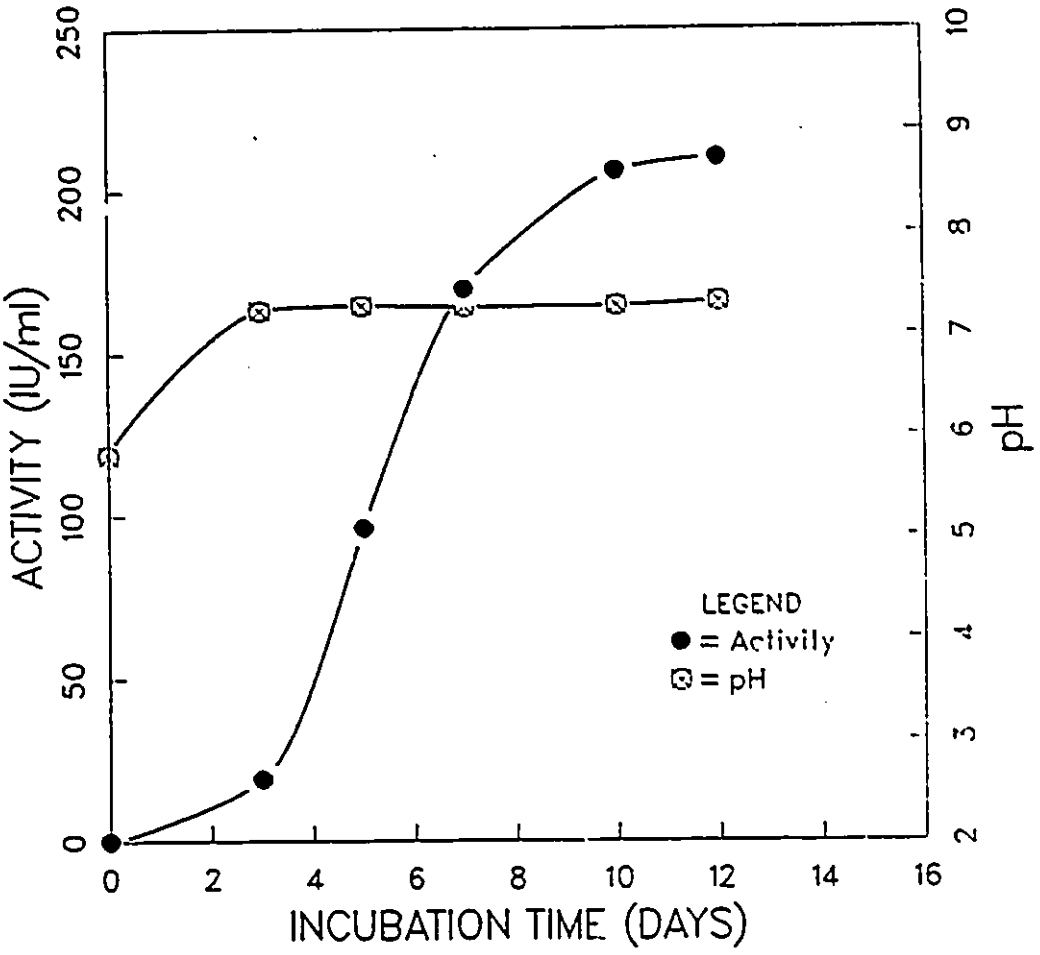

Figure 4.8: Kinetics of xylanase production and pH changes in medium containing 8% (w/v) canola meal

same regardless of the concentration of canola meal used. Also, similar pH increase has been observed when growing *T. reesei* QM 9414 in medium containing hemicellulose, or bagasse hemicellulose (Dekker, 1983).

The color of the cultures remained essentially the same throughout enzyme production, which was a dark brown due to the canola meal. Cultures containing higher amounts of canola meal were more viscous and slightly darker brown.

The results obtained for canola meal are different from those reported for other complex substrates. For example, using hydrolyzed wheat straw pulp at 1.5% concentration as a carbon source for *T. reesei* F-522, a maximum xylanase activity of 11.4 IU/ml was obtained after 8 days (Szczodrak, 1988).

The use of kraft paper mill sludge as a substrate for hydrolytic enzyme production has also been studied (Royer and Nakas, 1987). The study showed that *T. reesei* DAOM 167654 produced 8.7 IU/ml of xylanase activity after 7 days when grown in a 1% sludge solution. The best results were obtained using *T. longibrachiatum* giving 35.1 IU/ml of xylanase activity.

Ghosh and Deb (1988) used the fungus *Thielaviopis basicola* and produced 7.3, 12.0, 1.5, 7.0, and 7.2 IU/ml of xylanase activity in medium containing on xylan, rice straw, peanut shell, Sarkanda leaves and corn cob respectively. They obtained a maximum of xylanase activity of 34 IU/ml using 2% rice straw after 12 days.

The production of xylanase from *T. reesei* RUT C30 when grown on SED wood and SED extracted with water was 218 and 140 IU/ml respectively. For *T. harzianum* E58, activities of 350 and 450 IU/ml of xylanase respectively were acheived (Saddler *et al.*, 1985).

With the exception of the results obtained by Saddler *et al.* (1985) for *T. harzianum* E58, the xylanase activities obtained using canola meal were in most cases comparable to or better than those obtained by other workers on other raw materials. Although *T. harzianum* E58 produced high levels of xylanase activity, the disadvantage of this microorganism is it produces very little cellulase.

Despite the fact that the xylanase level in the medium with low Solka- floc concentrations was relatively high, this substrate is too expensive for industrial production of the enzyme. The same remark is found in the literature for cellulase production (Tangnu *et al.*, 1981). The results reported in this section show that *T. reesei* is able to use canola meal as a substrate for the production of xylanase, and it produced this enzyme in similar or better yields than from more expensive substrates such as Solka-floc, xylan or glucose. These results indicate that the use of canola meal as a substrate for industrial xylanase production could be feasible.

4.1.4 Effect of Culture Conditions on Xylanase Production

Bearing in mind that a complex interaction usually exists between the medium composition, pH, inoculum size and process conditions, the following subsection is devoted to the study of the effect of culture conditions on xylanase production using canola meal and in some cases Solka-floc as a carbon source. The results from this subsection will give an indication of the culture conditions which could be used to enhance xylanase enzyme production and as a starting point for later studies on scaling up this process. Some of the findings could also be of significance to industrial scale processes. Since canola meal concentrations of 8% were very difficult to work with because of rheological problems, subsequent tests were carried out using media with 4% canola meal, unless otherwise specified.

Effect of Marbles on Xylanase Production

A two percent Solka-floc media was used to test the effect of marbles on the production of xylanase by *T. reesei*. Marbles are generally used to break up the culture and enhance enzyme secretion. Figure 4.9 shows that there is a slight enhancement in xylanase production using 2% Solka-floc. After 12 days, a maximum xylanase activity of 172 IU/ml was achieved in the medium without marbles, this was higher than the

activity of 156 IU/ml achieved using marbles.

The culture was more filamentous and less pellotous when marbles were used, it was also a creamier green. These results suggest that xylanase secretion was better when *T. reesei* RUT30 was in an undisturbed pellet form compared to the form it is in after being broken up by the marbles. The use of marbles may cause partial damage to the biomass thereby lowering xylanase secretion. Therefore, marbles were not used in further tests except in those experiment comparing 2% cellulose and 2% Solka-floc as specified earlier (Subsection 4.1.1). The slight decrease in xylanase enzyme production observed due to the presence of marbles suggests that in a larger scale operation minimal agitiation would be more beneficial to xylanase production.

Effect of Tween 80 on Xylanase Production

The effect of Tween 80 on the production of xylanase was tested in a medium containing 4% canola meal. The concentration of Tween 80 was 0.2% v/v. The results in Figure figure:tgg1b show that the activity 128 IU/ml of culture suspension was obtained in the presence of Tween 80 while in the media without it the activity was only 72 IU/ml.

The mechanism of enhancement of xylanase production by Tween 80 is not known but it may be related to increasing the permeability of the cell membrane, thereby enabling more rapid enzyme secretion resulting in greater enzyme synthesis (Tangnu et al., 1981). Unfortunately, surfactants are expensive and can cause foaming problems in a full-scale process therefore the use of Tween 80 may be disadvantageous (Linko *et al.*, 1982). However, Tween 80 was successfully used in shake flasks cultures. This information suggests that although Tween 80 enhances xylanase production in shake flask cultures studies would have to be carried out to determine the optimal conditions for xylanase production in a full-scale process particularly with respect to surfactant cost and foaming problems.

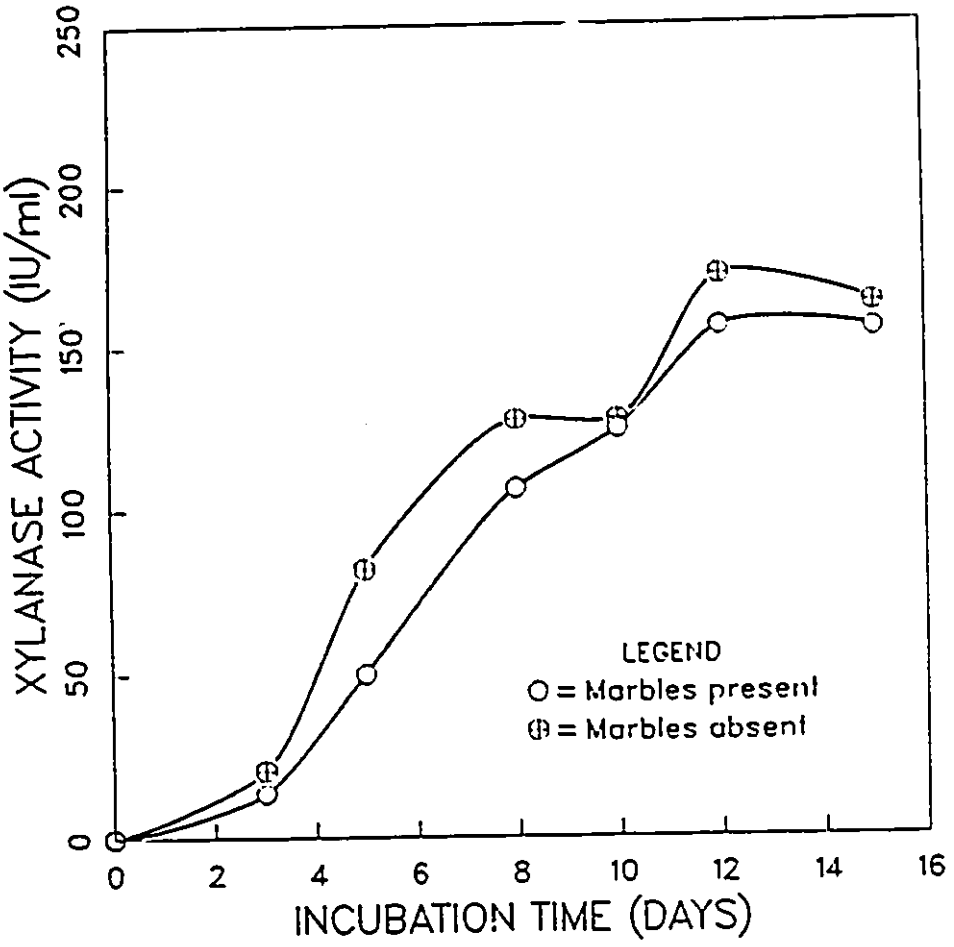

Figure 4.9: Effect of modification of fungal growth caused by marbles on xylanase production in medium containing 2% (w/v) Solka-floc as a carbon source

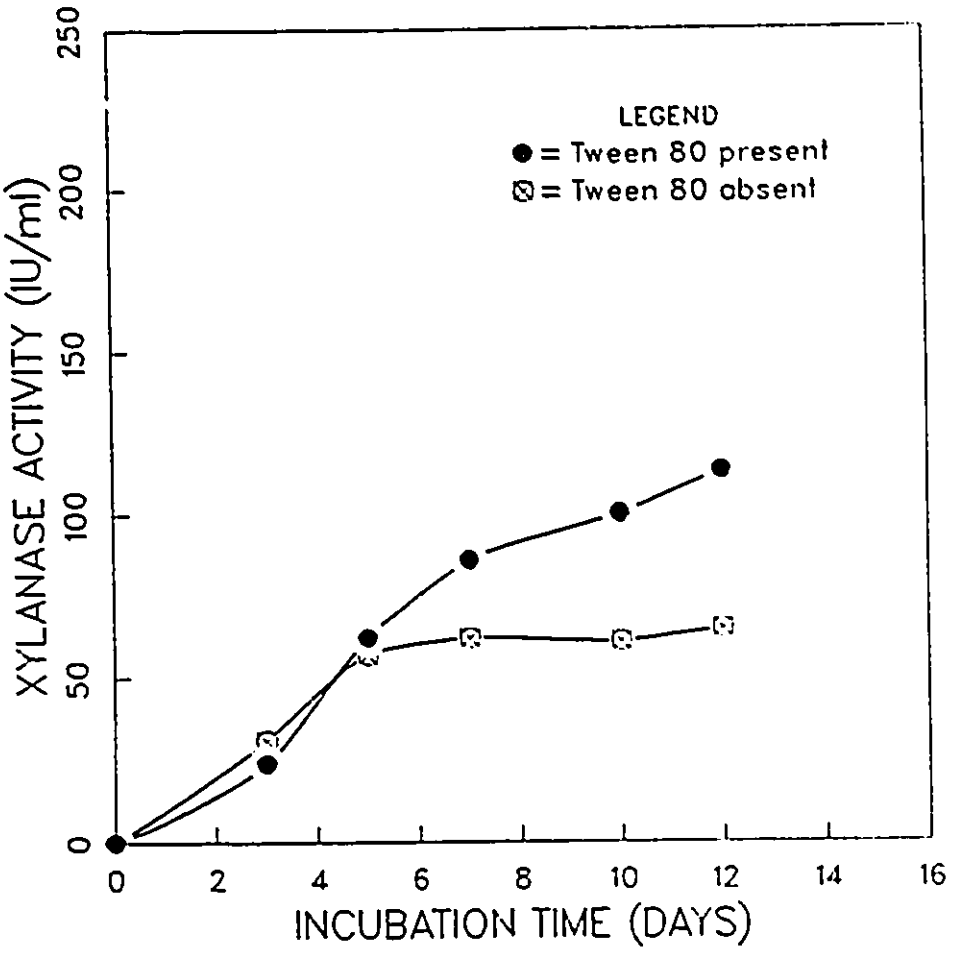

Figure 4.10: Effect of Tween 80 on xylanase production in a 4% (w/v) canola meal medium

Effect of Culture Temperature on Xylanase Production

Results in figure 4.11 show that culture temperature effects xylanse production. At 37°C a xylanase activity of 128 IU/ml was attained after 6 days. At the temperature of 28°C a maximum xylanase activity of 102 IU/ml occurred after 9 days of growth.

This is in agreement with conclusions made by earlier workers indicating that elevated temperatures enhance xylanase secretion in *T. reesei*. An explanation for the effects of temperature on induction and repression of extracellular carbohydrases is currently being investigated (Suh *et al.*, 1987).

Effect of Initial pH on Xylanase Production

The effect of initial pH on xylanase activity is shown in figure 4.12. The maximum xylanase production of 92 IU/ml was acheived in the medium with an initial pH of 4.7 after autoclaving. At the pH values of 3.6 and 5.6 the activities were only about 40% of that shown for pH 4.7. Irrespective of the initial pH value, in all cases the pH reached a plateau beiween 7.5 and 8.0 after 5 to 7 days of incubation.

Other workers have shown that the growth of *T. reesei* QM 9414 in buffered medium at pH 4.5 containing 1% bagasse or 1% unfractionated bagasse hemicellulose produced the highest level of hemicellulolytic enzyme activity (Dekker, 1983).

It has been shown that a temperature of 31°C and a pH of 4.5 for the first 48 h, then a temperature of 28°C and a pH maintained above 3.3 for the remaining fermentation time, were optimum for cellulase production in other strains of *T. reesei* (Tangu *et al.*, 1981). This reflects the extreme temperature and pH sensivity involved in determining optimal conditions for enzyme production.

Effect of Inoculum Size on Xylanase Production

The inoculum volume had little or no effect on xylanase formation (Figure 4.13). Maximum xylanase activities of 99, 114, and 100 were achieved between 9–11 days using 2.5,

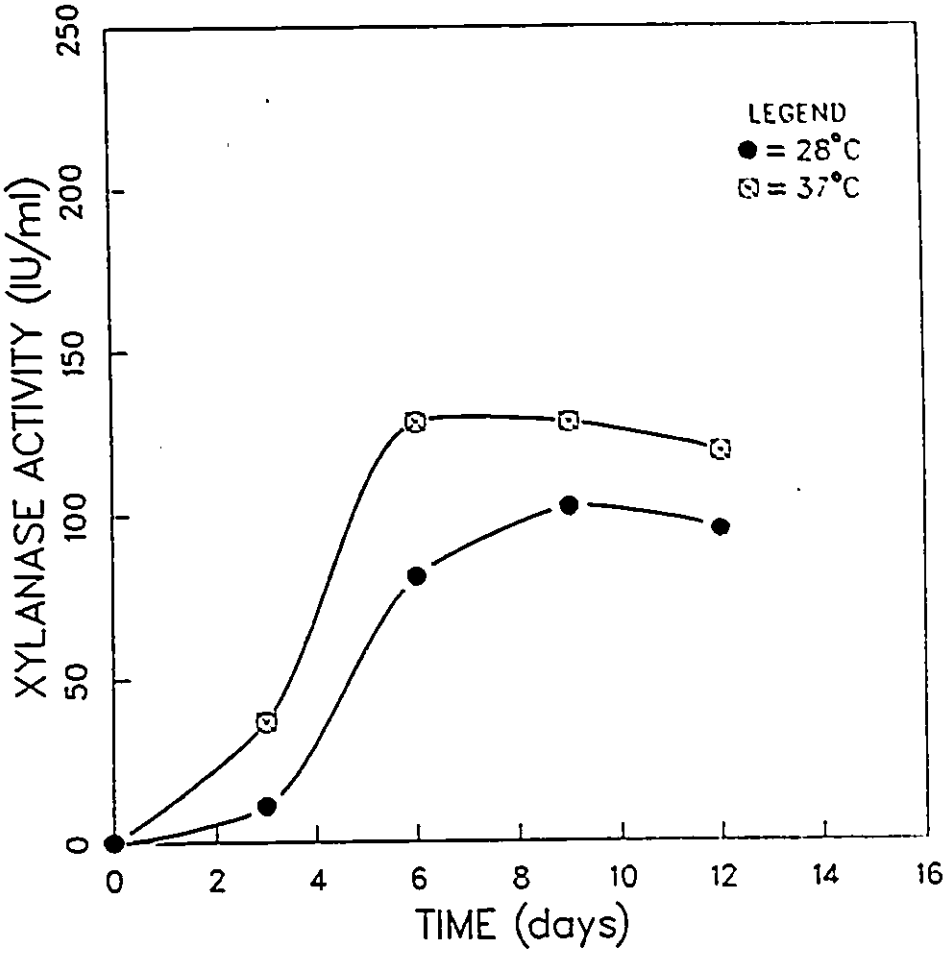

Figure 4.11: Effect of culture temperature on xylanase production in medium containing 4% (w/v) canola meal

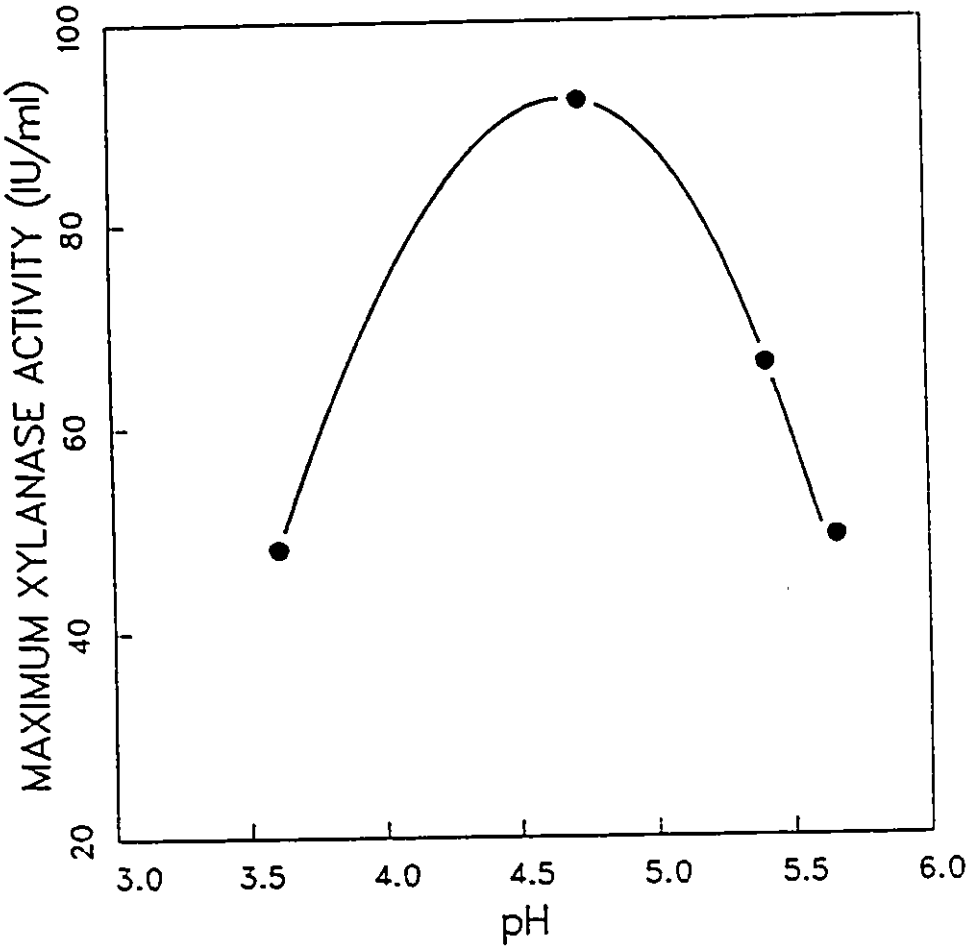

Figure 4.12: Effect of initial pH on maximum xylanase production in medium containing for a 4% (w/v) canola meal

5 and 10% (v/v) respectively of inoculum. This finding has important industrial significance because since industrial preparation of inoculum is costly, the reduced amount of inoculum needed for xylanase production will help to lower overall production costs of this enzyme.

4.2 Properties of the Xylanase Enzyme System Produced in Canola Meal or Solka-floc Media

The following section looks at some of the properties of the xylanase enzyme systems produced using canola meal or Solka-floc as carbon sources in the media.

4.2.1 Formation of Various Xylanolytic Activities in Different Media

Bearing in mind that the composition of media influences the amount and type of enzyme produced, various carbon sources were tested for the production of xylanolytic enzymes by *T. reesi* RUT C-30. The substrates used and enzymes produced by the fungus are shown in Table 4.1. The results show that in addition to xylanase, *T. reesei* produced β-xylosidase, acetyl-xylan esterase, and cellulase. In all these cases (except β-xylosidase activity for 1% (w/v) larchwood xylan), 1% glucose and 1% larchwood xylan resulted in activities lower than those obtained using the Solka-floc or canola meal as a substrate. Cellulase and β-xylosidase activities (except xylosidase for 5% canola meal) attained in the medium with canola meal were higher than those produced in using 1% Solka-floc. Eight percent canola meal contained slightly higher xylanase, and approximately 25% higher acetyl-xylan esterase activities as compared to those produced using Solka-floc (Table 4.1). The medium with 5% canola meal gave much lower xylanase activity than with 8% of the meal. But the addition of 0.25% (w/v) of Solka-floc or larchwood xylan to the medium with 5% canola meal enhanced the

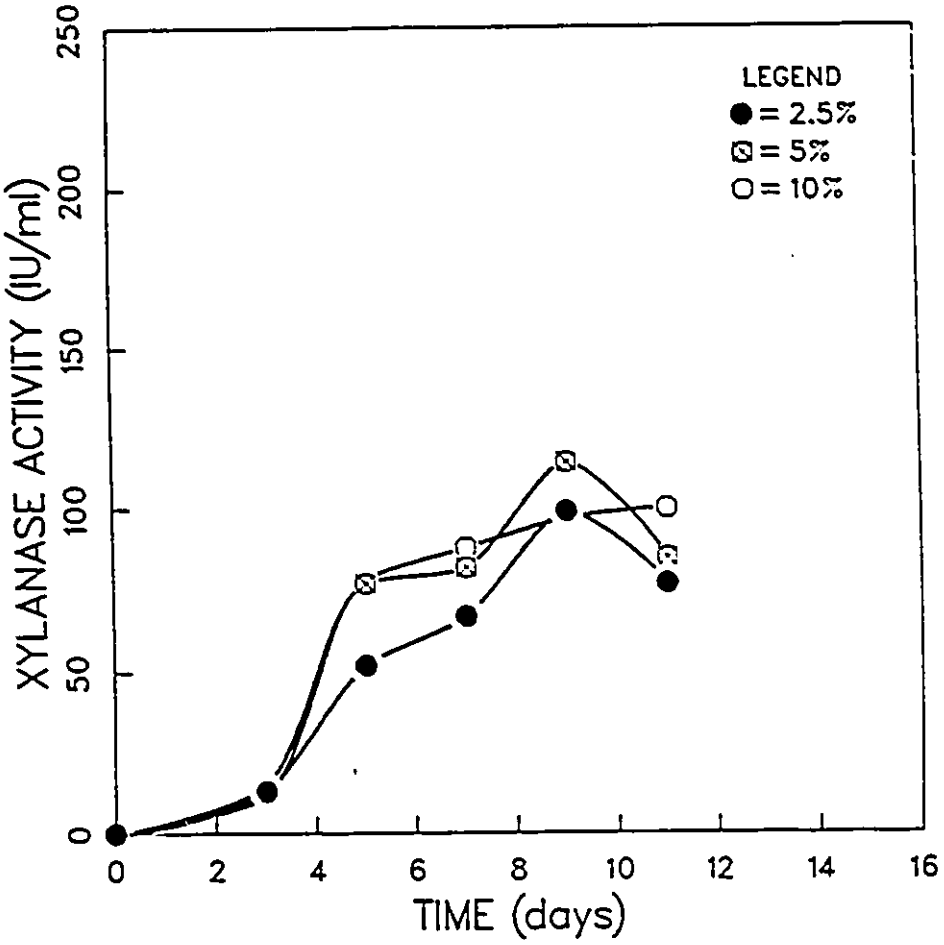

Figure 4.13: Effect of inoculum volume on xylanase production in a 4% (w/v) canola meal medium

Table 4.1: Xylanolytic activities in media containing different carbon sources, alone or in combination after a nine day incubation period

Substrate Used	Xylanase	β-Xylosidase	Acetyl-xylan Esterase (IU/ml)	Filter Paper Activity
1% Glucose	30	152	0.1	0.7
1% Larchwood Xylan (LWX)	117	1508	0.8	0.7
1% Solka-floc (SF)	207	979	1.9	1.8
5% Canola Meal (CM)	129	825	1.6	2.1
5% CM + 0.25% LWX	198	1088	1.1	3.1
5% CM + 0.25% SF	203	1200	1.8	2.9
8% Canola Meal	212	1435	2.1	3.0

enzyme activity almost to the level achieved using 8% canola meal. The addition of 0.25% (w/v) xylan and Solka-floc to 5% canola meal also raised the production of β-xylosidase, acetyl-xylan esterase, and cellulase closer to that of 8% (with the exception of acetyl-xylan esterase and cellulase for 5% canola meal plus 0.25% xylan) (Gattinger *et al.*, 1990*a*).

Activities such as acetyl-xylan esterase and β-xylosidase have been shown to act synergistically in the breakdown of xylan and enhance xylanase activity (Biely *et al.*, 1986; Dekker and Richard, 1976). Acetyl-xylan esterases are required to enhance the action of xylanase by removing o-acetyl residues (Biely *et al.*, 1986). Xylan occurs

naturally in a partially acetylated form and is associated with cellulose. Presence of cellulase in this system is helpful in breaking down cellulose associated with xylan in natural systems.

β-xylosidase, increases the degree of substrate hydrolysis in hemicellulolytic enzyme preparations, and has a role similar to that of β-glucosidase in the overall conversion of cellulose to glucose (Dekker, 1983). It is very important that the enzyme preparation used in the saccarification of heteroxylan contains not only a full complement of xylanase enzymes but also the required amount of β-xylosidase to ensure overall conversion to xylose. In a bioconversion process which converts heteroxylan into ethanol it is necessary that the final hydrolysis products are monosaccharides such as xylose which can easily be fermented into products such as ethanol. Most purified β-xylosidases showed no activity towards xylan.

The xylanase, xylosidase and acetyl-xylan esterase activites reported here are much higher than those reported by other researchers (Biely *et al.*, 1988, Dekker, 1983) using other cheaper raw materials such as sugar cane bagasse, and microorganisms such as, *T. reesei* strains QM9414 and MCG-77, and *Schizophyllum commune*. However, the cellulase activites produced in this study under conditions for xylanse production were much smaller than those reported for this strain (Ryu and Mandels, 1980).

4.2.2 Physicochemical Properties of the Canola Meal and Solka-floc Enzyme Systems

The pH optimum for the xylanase enzyme system produced on both Solka-floc and canola meal was determined to be approximately 5–6 (Figure 4.14). This is close to the results reported by other workers who found a pH optmum of 4–5 for *T. reesei* QM 9414 xylanase (Dekker, 1983), and approximately 6 for *T. reesei* $TD_1\beta 6$ xylanase (Durand *et al.*, 1984). These results show that in terms of pH optima, the enzyme system produced by *T. reesei* using inexpensive canola meal and more expensive Solka-floc as

substrates are essentially the same.

It is important to know the temperature optimum of an enzyme system in order to determine its temperature of maximal activity. Below the temperature optimum activity is controlled by diffusion and increases in temperature increase the speed of the molecules. Above the temperature optima the enzyme becomes denatured and a permanent loss in enzymatic activity occurs due to changes in enzyme structure. The temperature stability of an enzyme system is also important because the enzyme is not always stable at the temperature where it is most active. Therefore, both these factors must be considered when determining the temperature of operation of an enzymatic process.

The temperature optimum for both the enzyme systems produced on Solka-floc and canola meal was 50°C (Figure 4.15). This is in agreement with the results reported by Dekker (1983) who found the temperature optima to be between 55–60°C for *T. reesei* QM 9414. The enzyme produced by *T. harzianum* E58 had a similar temperature optimum of about 50°C (Saddler *et al.*, 1985), and the optimum temperature for *T. reesei* $TD_1\beta 6$ was 55°C (Durand *et al.*, 1984).

Both the enzyme systems produced on canola meal and Solka-floc showed a slight increase in activity after half an hour at 50°C (Figure 4.16) prior to an approximate 90% loss in enzyme activity after incubating the enzyme systems for 24 hours at 50°C. Similar slight increase in activity prior to the decrease shown in this figure has been observed for cellulase activity (Saddler *et al.*, 1985). The enzyme system produced using Solka-floc as a carbon source appears to be slightly more stable than that of the enzyme system produced using canola meal as substrate. After two weeks at 37°C, there was a 10% loss in activity in both the enzyme preparations. These preparations were much more stable at 4°C, they lost only about 10% of their activity after approximately 1 year. Although xylanases are generally quite labile, the enzyme preparations reported here are highly stable at 4°C and appear suitable for a long period of storage.

As a result of dialysis, the protein content of the enzyme system produced from

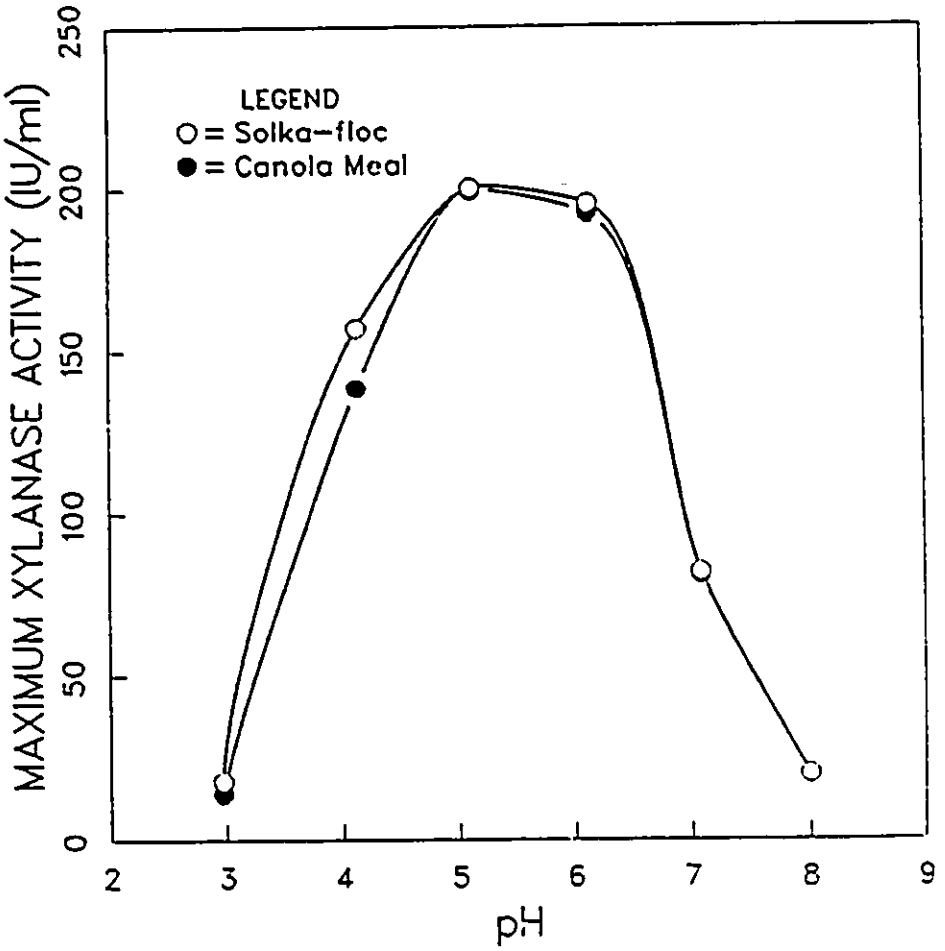

Figure 4.14: Effect of pH on the activity of the enzyme systems produced in canola meal or Solka-floc media

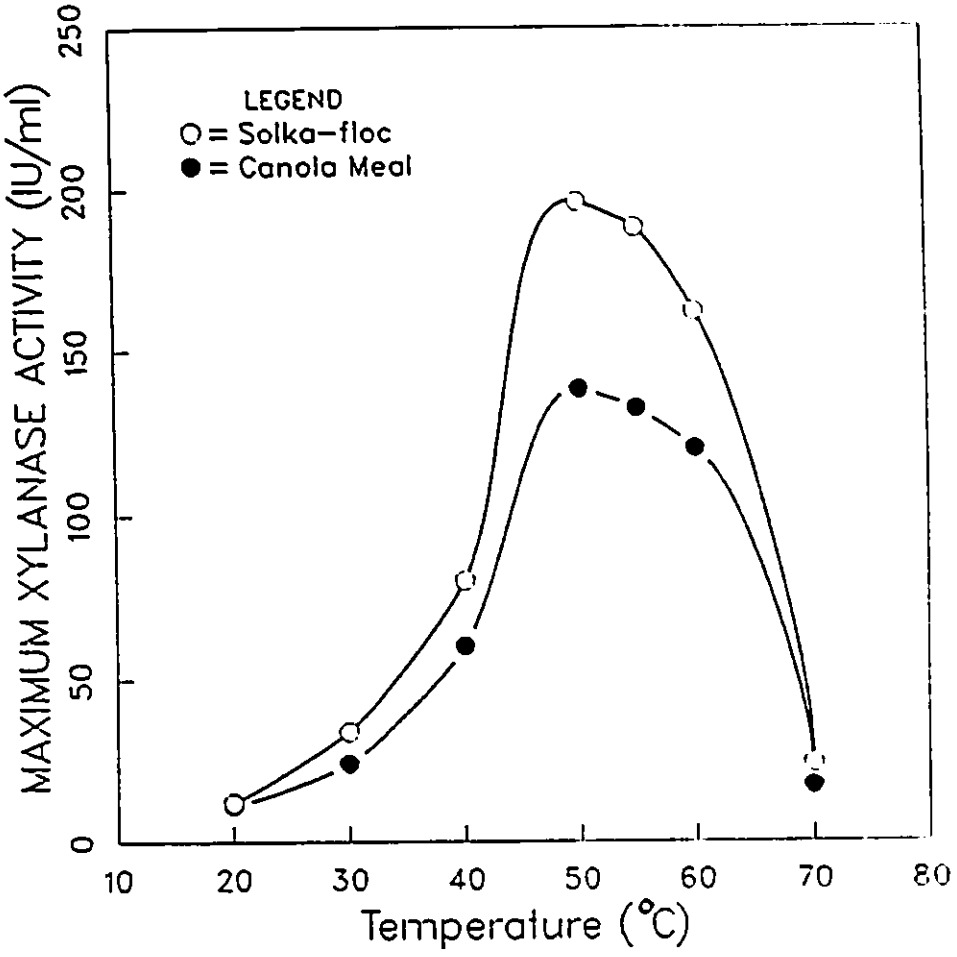

Figure 4.15: Effect of temperature on activity of the enzyme systems produced in canola meal or Solka-floc media

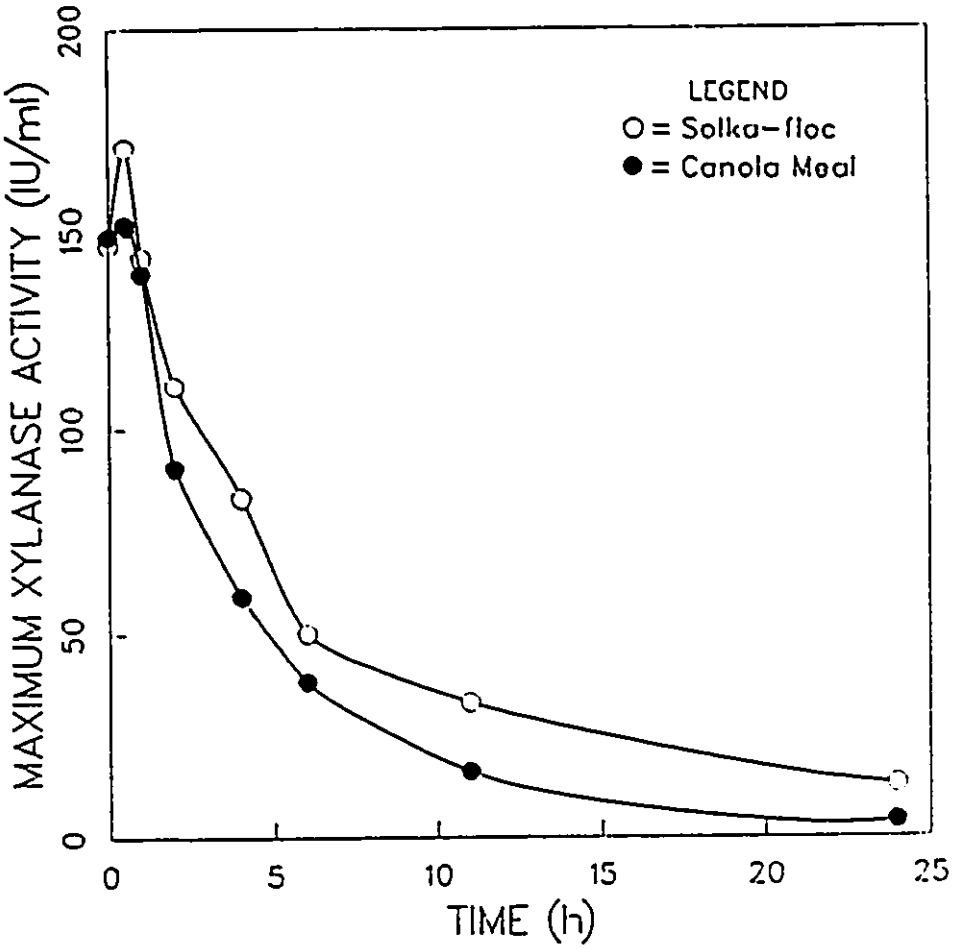

Figure 4.16: Stability at 50°C of the xylanase enzyme systems produced using canola meal or Solka-floc media

Table 4.2: Effect of dialysis on the enzyme systems produced using canola meal as a carbon source

Condition	Activity (IU/ml)	Protein (mg/ml)	Specific Activity (IU/mg)
Before Dialysis	98	5.22	18.8
After Dialysis	94	3.00	31.3

canola meal, decreased and caused the specific activity to increase by a factor of 2. On the other hand, there were only small changes in the protein as well as specific activity of the enzyme system produced from Solka-floc (Table 4.2 and Table 4.3). As canola meal contains about 40% (w/w) protein, peptides and other nitrogen constituents loss during dialysis appear to originate from the canola meal used as a substrate rather than loss of proteins produced by *T. reesei*.

A number of metal ions as well as other compounds were tested to determine their effect on the activity of the enzyme preparation made using canola meal as substrate (Table 4.4). Of the metal ions tested the most significant inhibition was caused by Hg^{2+} and Mn^{2+} ions, whereas slight stimulation was observed for the K^{+} and Ag^{+} ions. The compounds capable of binding or destroying the thiol groups and the reducing agents had inhibitory effect on enzyme activity (Table 4.4). Of the chelating agents tested, etheylenediaminetetraacetic acid and nitrilotriacetic acid caused inhibition to the greatest extent suggesting that divalent ions such as Ca^{2+} and Mg^{2+} ions are necessary for the enzyme to function. Slight inhibition was also caused by NaN_3 and

Table 4.3: Effect of dialysis on the enzyme systems produced using Solka-floc as a carbon source

Condition	Activity (IU/ml)	Protein (mg/ml)	Specific Activity (IU/mg)
Before Dialysis	134	2.61	51.3
After Dialysis	110	2.00	55.0

N-ethylemaleimide suggesting that SH groups are potentially involved in the enzymatic mechanism.

The results reported in section 4.2 show that in terms of the physical properties tested, the enzyme system produced using canola meal as a carbon source is very similar to the one using Solka-floc which is the more expensive substrate.

4.3 Applications of the Xylanase Enzyme System

One of the purposes of this section is to compare the ability of the enzyme systems produced using Solka-floc and canola meal media to hydrolyse various xylan containing materials. A second purpose of this section is to study the temperature stability of some of the enzymes involved in this hydrolysis.

Table 4.4: Effect of various compounds on crude and dialysed xylanase preparation produced in canola meal medium

Compound Tested	Concentration (mM)	Change in Activity Relative to Control
Metal Ions		
KCN (K^+)	10	+10%
$HgCl_2$ (Hg^{2+})	10	-95%
$AgNO_3$ (Ag^+)	1	+7%
$ZnCl_2$ (Zn^{2+})	1	-10%
$MnSO_4$ (Mn^{2+})	10	-62%
$CoCl_2$ (Co^{2+})	10	-11%
$CaCl_2$ (Ca^{2+})	10	-5%
$MgCl_2$ (Mg^{2+})	10	-12%
Thiol and Reducing Agents		
Glutathione	10	-10%
Cysteine	10	-5%
Dithiothreitol	10	-12%
Ascorbic Acid	10	-25%
Chelating Compounds (Samples Undialysed)		
Ethylenediaminetetraacetic Acid	10	-20%
Sodium Azide	10	-11%
Nitrilotriacetic Acid	10	-19%
N-ethylemaleimide	10	-7%

4.3.1 Hydrolysis of Agricultural Substrates

The abilities of the enzyme systems produced by *T. reesei*, in media containing canola meal and Solka-floc as the sole carbon source, to hydrolyze some agricultural materials are compared in Table 4.5. These results show the extent of saccharification in 24 hours. After that time the reaction did not proceed any further. In these tests, both of the enzyme systems were adjusted to contain 140 IU of xylanse/100 mg of substrate. As no attempts were made to adjust the concentration of secondary enzyme activities such as acetyl-xylan esterase, β-xylosidase, and cellulase, the enzyme preparations slightly differed in these activities. The degree of saccharification attained using the enzyme preparation produced from canola meal was higher in the case of canola meal, corn bran, and wheat bran as compared to the saccharification achieved with the enzyme preparation produced from Solka-floc. When corn cob, straw and larchwood xylan were used as the substrate for saccharification the enzyme systems from both sources showed the same activities (Gattinger *et al.*, 1990*a*).

These results are in agreement with those of Khan *et al.* (1989*a*) who achieved 21%, 32%, and 82% hydrolysis of sugars in cornbran, wheat bran, and larchwood xylan respectively, using enzyme from *T. reesei* cultivated on Solka-floc in a fermentor.

These results show that canola meal can be used successfully to replace more expensive substrates in the production of xylanase without affecting the saccharification ability of the produced enzyme system by *T. reesei*. The hydrolysis of canola meal by this enzyme system is of particular interest in converting this surplus material to fermentable sugars that can be further processed to expensive end products such as solvents and chemicals by known fermentation processes.

Table 4.5: Saccharification of various xylan containing materials with the enzyme systems produced by *T. reesei* using Solka-floc and canola meal as a carbon source

	Xylanase Produced on Solka-floc		Xylanase Produced on Canola Meal	
	Saccharification[a] (%) based on			
Substrate	Weight of substrate	Sugar content in substrate	Weight of substrate	Sugar content in substrate
Canola Meal[b]	10	34	12	42
Corn Bran[c]	9	19	10	21
Corn Cob	30	-	30	
Straw	19	-	19	
Wheat Bran[c]	26	37	30	43
Larchwood Xylan	79	83	78	82

[a]Saccharification time was 24 hrs

[b]Canola meal contained 28% polysaccharides

[c]Corn and wheat brans contained 48% and 70% polysaccharides respectively

4.3.2 Kinetics of Hydrolysis of Xylan, Canola Meal and Corn Bran at 50°C

The results from Subsection 4.3.1 showed that the enzyme system produced using canola meal was more or equally efficient as compared to that produced using Solka-floc in hydrolyzing canola meal, corn cobs, corn and wheat brans, straw, and larchwood xylan to fermentable sugars. The purpose of this subsection is to determine whether there are any differences in the kinetics of hydrolysis of larchwood xylan, corn bran and canola meal (Figures 4.17, 4.18 and 4.19) between the enzyme systems produced using canola meal and Solka-floc as carbon sources. The substrate xylan was chosen for the test because this is the standard for the assay of xylanase enzyme activities. Corn bran was chosen because it is very difficult to hydrolyse. Canola meal was chosen to compare the abilities of the enzyme system produced using Solka-floc and canola meal to hydrolyse canola meal and to see if the enzyme system produced using canola meal as substrate was better able to hydrolyse canola meal. In these three tests, 140 IU of xylanase per 100 mg of substrate was used.

The enzyme systems produced using Solka-floc and canola meal were equally efficient in hydrolyzing larchwood xylan (Figure 4.17). The saccharification based on the weight of substrate was 79 and 78% for the Solka-floc and canola meal systems respectively.

The enzyme system produced using canola meal was essentially the same as that for Solka-floc in hydrolyzing corn bran, giving 21 and 19% hydrolysis of total sugars respectively (Figure 4.18).

The kinetics of hydrolysis of canola meal with the enzyme systems produced on canola meal and Solka-floc are shown in Figure 4.19. The results show an advantage of the canola meal enzyme system in the saccharification of this substrate, giving 42% compared to 34% hydrolysis by the enzyme system produced on Solka-floc. This may be due to the presence of higher proportions of specific enzymes required for the hydrolysis

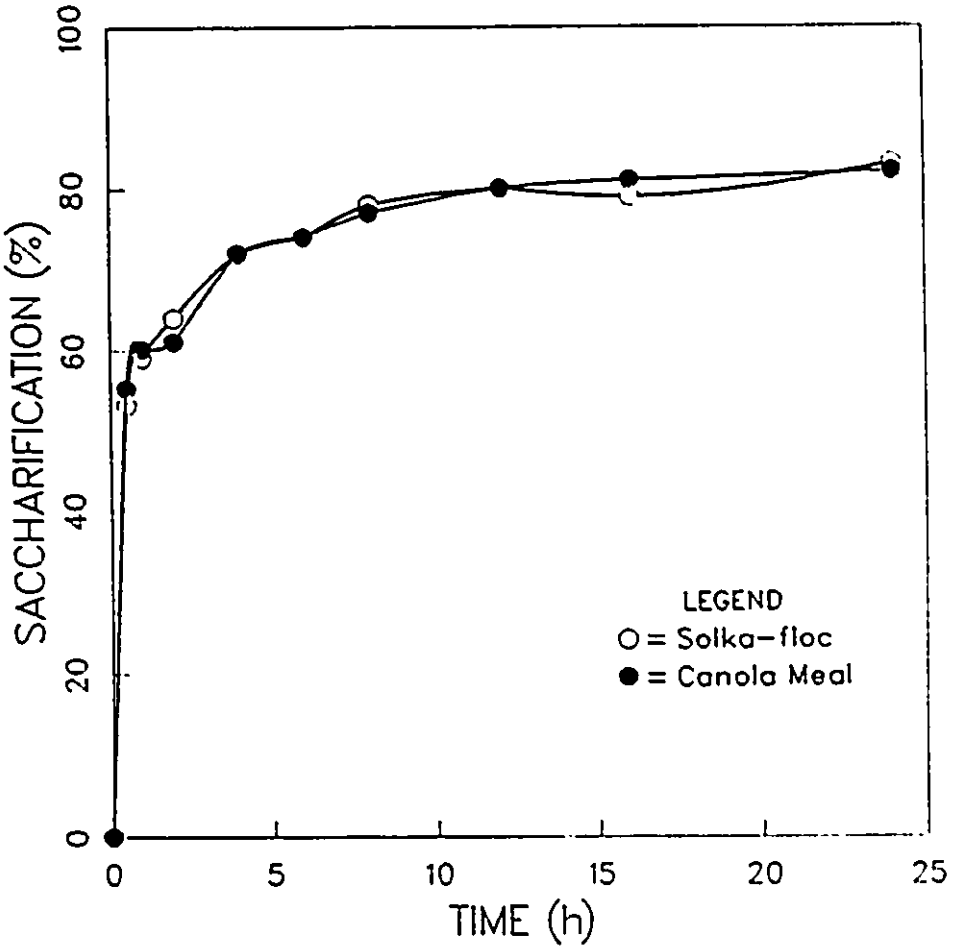

Figure 4.17: The kinetics of hydrolysis of larchwood xylan at 50°C by the enzyme systems produced on canola meal and solka-floc

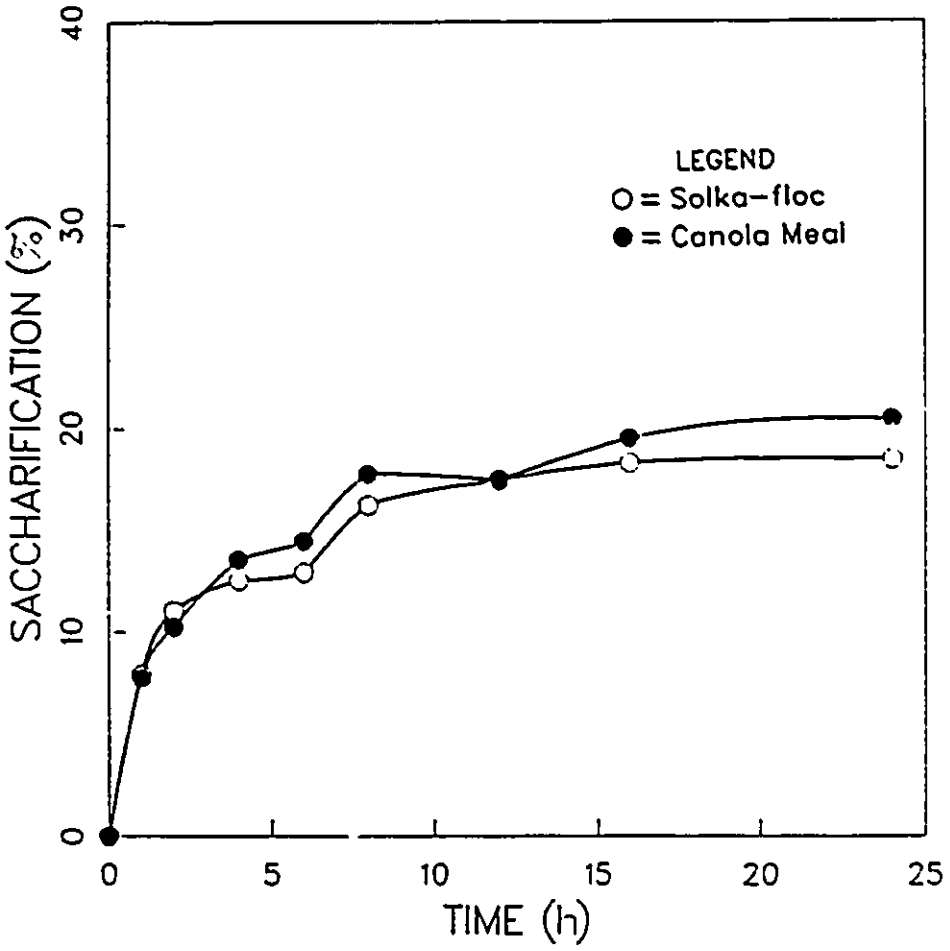

Figure 4.18: The kinetics of hydrolysis of corn bran at 50°C by the enzyme systems produced on canola meal and Solka-floc

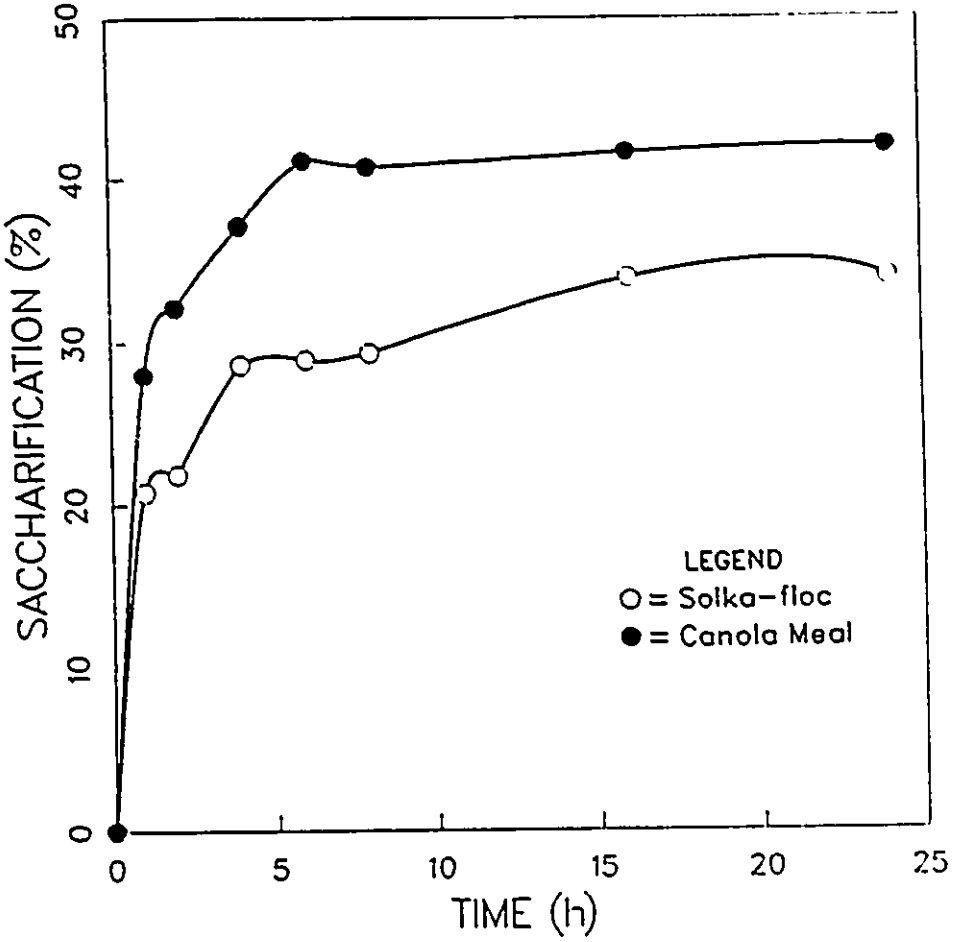

Figure 4.19: The kinetics of hydrolysis of canola meal at 50°C by the enzyme systems produced on canola meal and Solka-floc

of canola meal and produced through induction. The incomplete hydrolysis of canola meal is probably due to enzyme instabilty from exposure to high temperatures for prolonged times, but end-product inhibition of enzyme or the chemical nature of the substrates *ie.* degree and frequency of branched substituents along the xylan chain may also affect hydrolysis (Dekker, 1983).

Comparing the results from these three Figures with those of Table 3 it can be seen that in all cases the hydrolysis (based on final values) was over 70% complete after 4 hours and over 85% complete after 8 hours. During the initial saccharification period, the most easily hydrolyzable bonds in the substrate are hydrolyzed and the effects of adverse conditions such as end-product inhibition and enzyme denaturation are minimal. For practical applications, saccharification values are best determined using the specific substrate under the conditions of the process (Morisset and Khan, 1984).

4.3.3 A More Detailed Study into the Enzymatic Hydrolysis of Canola Meal

Canola meal contains about 28% polysaccharides comprised of arabinogalactan, arabinan, xylan, xyloglucan (amyloid) and cellulose as the major constituents (Siddiqui and Wood, 1977), while the starch is present only in a small amount (Blair and Reichert, 1984). The main sugars obtained as a result of hydrolysis of the dehulled, oil-free meal are: arabinose, galctose, glucose and xylose (Theander and Aman, 1977 and 1978). In canola meal these polysaccharides originate from the fibrous materials present in the seed coat and in the cotyledon wall. It has been recognized that the removal of fiberous materials from the canola meal is desireable to increase the availablilty and utilization of the proteins and the trace metals present in canola meal containing feeds (Shires *et al.*, 1983). One method for converting and upgrading this product is by degrading the fibrous materials to fermentable sugars through the use of enzymes.

Having produced enzyme systems using canola meal and Solka-floc as carbon sources which are both capable of saccharifying canola meal to some extent, it was decided that investigation into various aspects involved in the enzymatic saccharification of canola meal was merited. This subsection studies the hydrolysis of canola meal to fermentable sugars by the use of the *T. reesei* enzyme system and compares its hydrolysis with some commercially available hemicellulases. In all cases, an enzyme substrate ratio was chosen to ensure the enzyme was in excess and that maximum canola meal hydrolysis occurred. This ratio was determined as follows.

Determination of the Minimum Amount of Enzyme Activity Needed for Maximum Canola Meal Saccharification

The relation between enzyme concentration and substrate weight for maximum saccharification is shown in Figure 4.20. Since the saccharification of canola meal appears to occur as a result of multiple enzyme action, and the ratio between various activites also varied from one preparation to another, no single activity was found suitable for use as as criterion of saccharifying ability. The enzyme requirement for hydrolyzing a given weight of substrate was determined by varying the enzyme volume to substrate ratio. The enzyme capable of causing maximum saccharification, and rendering substrate unhydrolysable by fresh enzyme was considered optimum, this value was 1400 IU of xylanase per gram of canola meal substrate. In terms of xylanase activity, 140 IU of xylanase activity corresponded to 1 ml of enzyme in this case.

In order to improve saccharification ability of enzyme preparations it is not sufficient to increase the enzyme concentration, but it is also necessary to eliminate end-product inhibition, enzyme denaturation, the resistance of substrate to enzymatic action through pretreatment and other inhibitory factors (Morisset and Khan, 1984).

Addition of fresh enzyme to previously saccharified and washed canola meal substrate did not cause further sugar production. The enzyme was added at a ratio of

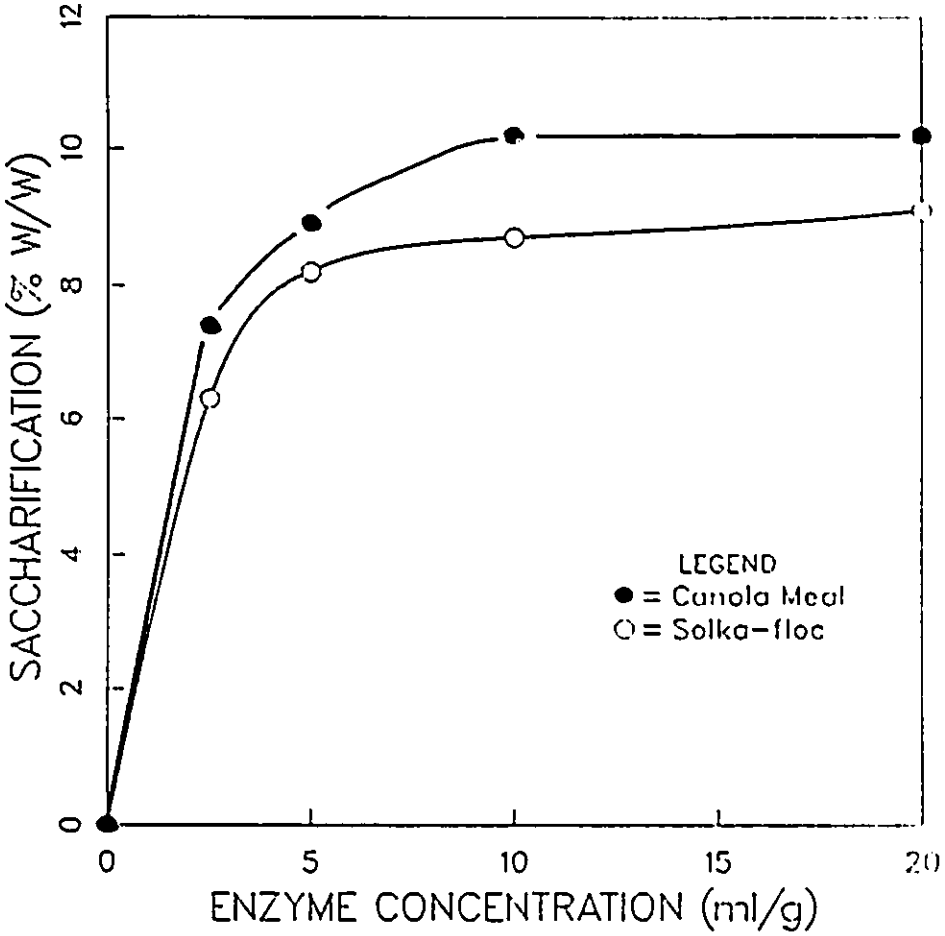

Figure 4.20: Effect of enzyme preparation concentration on saccharification of canola meal over 24 hours

140 IU xylanase activity per 100 mg canola meal substrate. This shows that all the substrate which could be hydrolyzed by the enzyme was converted to sugars over the 24 hour time period. It also shows that the initial substrate:enzyme ratio was sufficient to cause over 95% of the total possible hydrolysis, since fresh enzyme was not capable of causing further hydrolysis.

Kinetics of Hydrolysis of Canola Meal at 37°C and 50°C Using the Enzyme Systems Produced on Solka-floc and Canola Meal

Figure 4.21 shows the kinetics of hydrolysis of canola meal with the enzyme system produced on canola meal and Solka-floc at 37 and 50°C. In all cases, maximum saccharification was achieved in 24 hours, however the rate of hydrolysis at 37°C was slightly slower than at 50°C. Saddler *et al.* (1985) also noted that the extent of hydrolysis was less at lower temperatures. The similar trend was noticed by Durand *et al.* (1984) who found that hydrolysis of wheat bran, corn straw and beet pulp all decreased when hydrolysis was carried out at 50°C relative to 60°C. For longer term hydrolysis the half-life of the enzyme at elevated temperatures has to be considered, optimum conditions must be determined with respect to extent and rate of hydrolysis and enzyme stability.

Table 4.6 shows the sugars in canola meal (Theander and Aman, 1977). According to this table xylose constitutes approximately 13% of the total sugars in canola meal. These results suggest that in addition to xylanases a variety of other enzymes are necessary to degrade canol meal. An investigation into the enzymes present from the canola meal and Solka-floc enzyme preparations and their temperature stability will be discussed later in this subsection.

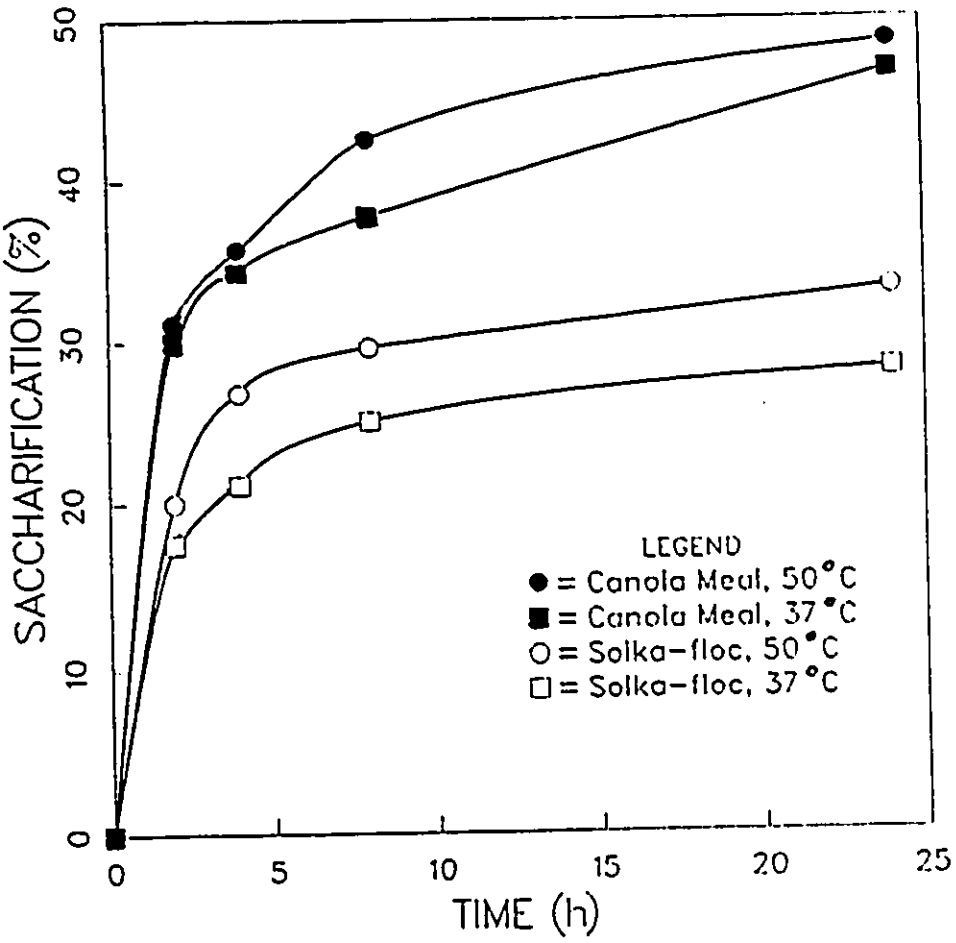

Figure 4.21: The kinetics of hydrolysis of canola meal at 37° and 50°C using the enzyme systems produced on Solka-floc and canola meal

Table 4.6: Sugars in canola meal (Theander and Aman, 1977)

Sugar	% of Total sugar content
Arabinose	27.4
Fucose	1.6
Galactose	24.3
Glucose	29.5
Mannose	2.1
Rhamnose	1.6
Xylose	13.3

Effect of Steam Sterilization on the Enzymatic Hydrolysis of Canola Meal

In order to get more insight into the ability of enzyme preparations from *T. reesei* to saccharify canola meal, a third enzymatic preparation was also utilized. The preparation was obtained using Solka-floc as a carbon source, but the cultures were grown in a fermentor. The commercial preparations Gamanase and Novozym were also used as a basis of comparison with the *T. reesei* preparations.

The purpose of pretreating lignocellulosic substrates before enzymic hydrolysis is to render the material maximally susceptible to attack by the polysaccharide degrading enzymes. Many different types of pretreatment have been studied. For example, chemical (with alkali or acid) mechanical (compression or ball milling), radiation (Kumakura and Kaetsu, 1988), and thermal (steam explosion) pretreatment for example the Iotech process (Sinitsyn *et al.*, 1983) are all methods presently used. For wheat pretreatment steam explosion was the best, followed by defibration, than chemical treatment with H_2O_2 (Vallander and Eriksson, 1985). Various substrates used in these tests were steam sterilized before use in order to minimize changes caused by microbial contamination during hydrolysis. The effects of steam sterilization were investigated for canola meal.

For the enzymatic hydrolysis of canola meal, a pretreatment consisting of autoclaving at 121°C for 15 min appears necessary (Table 4.7). Without this pretreatment, only about 2–3% (w/w) of the canola meal was hydrolyzed by enzyme preparations from *T. reesei*, or by Gamanase and Novozym. As a result of this pretreatment, the amount of water-soluble carbohydrates which corresponds to 8.1% of canola meal was released. The released water-soluble carbohydrate fraction contained about 4% free sugars and about 96% water soluble polysaccharides. These polysaccharides were hydrolyzable by hydrochloric acid to free sugars. The liquid chromatographic analysis of these water soluble polysaccharides after acid hydrolysis showed that they contain arabinose and glucose as the major constituents, and galactose as a minor constituent (Figure 4.22). These polysaccharides will be arbitrarily termed as arabinoglucans. The Gamanase

and the Novozyme enzyme preparations had the ability to hydrolyse these arabinoglucan polysaccharides, but even the cultivation of *T. reesei* in medium containing canola meal and Solka-floc failed to induce the formation of enzymes that could hydrolyse these polysaccharides.

From autoclaved canola meal, the *T. reesei* enzyme system produced on canola meal, Solka-floc and Solka-floc in a fermentor respectively, released 21.5%, 18.0% and 20.0% g (w/w) of total sugars, and 13.6%, 9.9% and 12.1% g (w/w) of free sugars. While from autoclaved canola meal the Gamanase and Novozym preparations respectively released 15.4% and 18.6% g (w/w) of both total and free sugars. Considering that canola meal contains only approximately 28% (w/w) polysaccharides, the enzymes from the *T. reesei* enzymes system are capable of releasing at least 65% of the total sugars available. The increased level of free sugars produced by the Gamanase and Novozym preparations, compared to that produced by *T. reesei*, was mainly due to their ability to hydrolyse the polysaccharides (arabinoglucans) released from canola meal as a result of autoclaving. Since the *T. reesei* enzyme system did not hydrolyse soluble arabinoglucans, the higher amounts of total sugars produced by the enzyme preparations from *T. reesei* indicates that this enzyme system hydrolyzed more insoluble polysaccharides present in the canola meal than Gamanase or Novozym preparations (Gattinger *et al.*, 1990*b*).

4.3.4 A Study of the Enzymes Involved in the Enzymatic Saccharification of Canola Meal

The following subsection continues the investigation into the enzymatic hydrolysis of canola meal, but in this case the focus is on a detailed look into the enzymes involved in the hydrolysis.

Table 4.7: Effect of autoclaving on canola meal, and on its hydrolysis by *Trichoderma reesei* and two commercial enzyme preparations incubated overnight at 50°C

Treatment	Enzyme preparation used	Water soluble sugars released (%, ww^{-1})		
		Free (1)	Total[a] (2)	Polysaccharides (2) (1)
Non-autoclaved	None (control)	<0.1	<0.1	<0.1
Autoclaved	None (control)	0.3	8.1	7.8
Non-autoclaved	*T. reesei*[b]	1.3	3.1	1.8
Autoclaved	*T. reesei*[b]	13.6	21.5	7.9
Non-autoclaved	*T. reesei*[c]	1.0	1.7	0.7
Autoclaved	*T. reesei*[c]	9.9	18.0	8.1
Non-autoclaved	*T. reesei*[d]	1.1	2.2	1.1
Autoclaved	*T. reesei*[d]	12.1	20.0	7.9
Non-autoclaved	Gamanase	1.7	3.0	1.3
Autoclaved	Gamanase	15.4	15.4	<0.1
Non-autoclaved	Novozym	2.1	3.4	1.3
Autoclaved	Novozym	18.6	18.6	<0.1

[a] After hydrolysis with hydrochloric acid

[b] *Trichoderma reesei* was cultivated in shake flasks in medium containing canola meal as a carbon source

[c] *Trichoderma reesei* was cultivated in shake flasks in medium containing Solka-floc as a carbon source

[d] *Trichoderma reesei* was cultivated in a fermentor in medium containing Solka-floc as a carbon source

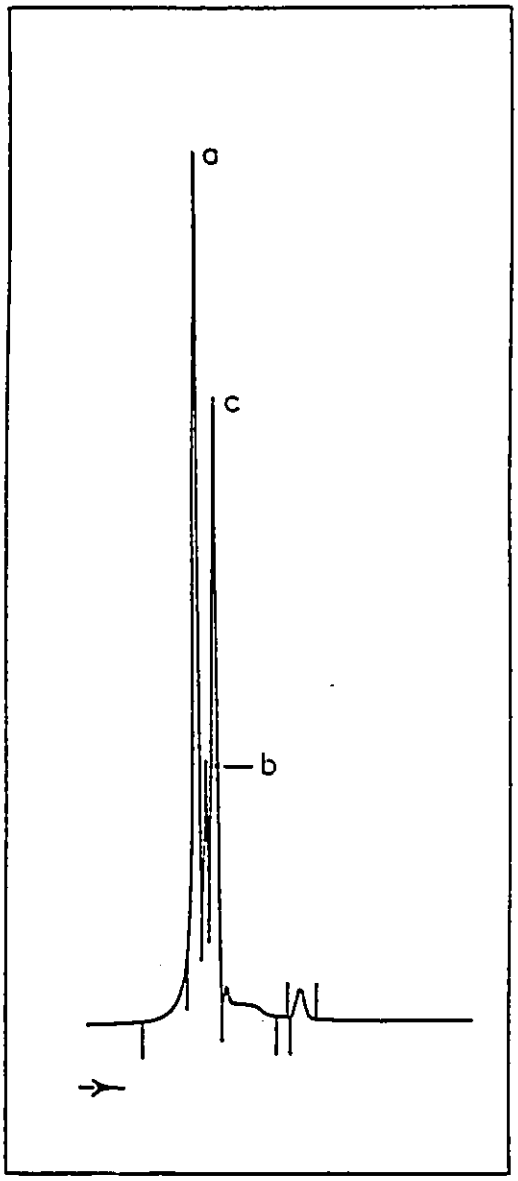

Figure 4.22: Products of hydrolysis of the polysaccharide produced after steam sterilization of canola meal: a glucose; b galactose; and c arabinose

Specific Activities of Various Enzyme Preparations Used for the Hydrolysis of Canola Meal

The enzyme profile of various enzyme preparations used in this study is given in Table 4.8. The enzyme preparations obtained from *T. reesei* were richer in arabinosidase, cellulase, glucosidase, mannosidase, xylanase and xylosidase activities compared to the Gamanase or Novozyme preparations.

In hemicellulase activities, *T. reesei* preparations from Solka-floc were most active in hydrolyzing galactomannan while Gamanase and Novozyme preparations in hydrolyzing arabinoglucan. On the other hand, enzyme preparations from *T. reesei* had little or no activity to hydrolyze arabinoglucan, but had approximately the same activity to hydrolyze arabinogalactan, galactan, β-glucan as the Gamanase or Novozym preparations. The Gamanase and Novozym enzyme preparations were better able to hydrolyze 1:3α glucans (starch), while the Novozym preparations had higher galactosidase and 1:3βglucanase activities (Gattinger *et al.*, 1990*b*).

One interesting point to note is the specific xylanase activities in the commercial preparations are significantly lower than those produced using canola meal and Solka-floc as substrates in shake flasks. This shows that in this case some activities acheived in this work were much better than those obtained industrially.

Effect of Heating on Cellulase, Hemicellulase and Glucanase Activities

Exposure of the five enzyme preparations used in this study to 50° for 24 h before use had little or no deleterious effect on hemicellulase activities (with the exception of galactomannanase produced from Solka-floc in shake flasks) (Table 4.9). There was also little or no loss in galactosidase, mannosidase (with the exception of the enzyme systems produced on Solka-floc and canola meal in shake flasks), β- glucanase activity (except gamanase). The heat exposure caused over 84% loss in cellulase, β-xylanase of the enzyme systems produced from Solka-floc and canola meal in shake flasks xylosidase

Table 4.8: Specific activities of various enzyme preparations used for the hydrolysis of canola meal

Enzyme activity	Substrate used	*T. reesei*[a]	*T. reesei*[b]	*T. reesei*[c]	Gamanase	Novozym
		IU/mg protein				
Arabinosidase	4NP[d] Arabinopyranoside	222	286	302.0	3.1	26.0
Cellulase	Filter paper	0.8	0.0	1.0	0.1	0.5
Galactosidase	4NP Galactopyranoside	3.2	14.7	7.6	2.1	103.1
1:3α Glucanase	Starch	0.2	0.4	0.1	4.3	5.9
1:3β Glucanase	Laminarin	0.1	0.3	0.1	0.1	0.8
1:4β Glucanase	Carboxymethyl Cellulose	0.1	0.3	0.5	0.2	0.3
β Glucanase	β-Glucan (from barley)	0.1	0.2	0.1	0.1	0.1
Glucosidase	4NP Glucopyranoside	347	674	83.0	62.9	40.8
Hemicellulase	Arabinogalactan	0.01	0.02	0.01	0.01	0.02
Hemicellulase	Arabinoglucan (Canola meal)	0.02	0.05	0.02	0.24	1.29
Hemicellulase	Galactan	0.02	0.03	0.02	0.02	0.02
Hemicellulase	Galactomannan (Locust beans)	0.24	0.64	0.44	0.33	0.11
Mannosidase	4NP Mannopyranside	1.7	3.7	6.5	0.7	0.6
β-Xylanase	Xylan (Oats spelts)	37.9	82.5	9.3	1.6	3.8
Xylosidase	4NP Xylopyranoside	417	6510	499.3	10.0	122.1

[a] *Trichoderma reesei* was cultivated in shake flasks in medium containing canola meal as a carbon source

[b] *Trichoderma reesei* was cultivated in shake flasks in medium containing Solka-floc as a carbon source

[c] *Trichoderma reesei* was cultivated in a fermentor in medium containing Solka-floc as a carbon source

[d] 4NP=p-nitrophenyl

activites. The loss in the majority of the remaining activities tested was between 20-80%.

These results show that cellulase and xylanase activities are relatively unstable at 50°C and agree with earlier published data (Dekker, 1983; Durand *et al.*, 1984), while the hemicellulase activities that cause the hydrolysis of arabinogalactan, galactomannan, galactan, or arabinoglucan are relatively stable.

The saccharifiction of canola meal is brought about by the action of a multiple enzyme system, the hemicellulases of which are most important. These tests shows that about 4% (w/w) hydrolysis of canola meal was accomplished by the action of cellulase, about 1% (w/w) by the action of xylanase and over 16% (w/w) by the action of hemicellulases. However the exact nature of the hemicellulases involved is difficult to demonstrate at the present time because: (i) there is little or no information available in the literature on hemicellulases other than xylanase, and (ii) the unavailability of specific substrates to assess the activity of various hemicellulases and the nature of their attack (Gattinger *et al.*, 1990*b*).

Hydrolysis of Canola Meal By Enzyme Preparations Used Before and After Overnight Exposure to 50°C

Results of the tests made to compare the hydrolysis of canola meal with enzyme preparations used before and after their exposure to 50°C for 24 h indicated that hydrolysis occurred mainly as a result of hemicellulases (Table 4.10). Loss of cellulase and β-xylanase activities in the enzyme preparations from *T. reesei* as a result of exposure to 50°C temperature, lowered the hydrolysis of the meal by 4.2–6.0%, while the loss of only xylanase in the Gamanase preparation, which originally possessed little or no cellulase activity, caused a decrease of a little over 1%. It appears that hydrolysis of polysaccharides present in the canola meal occurred as a result of the combined action of a number of hemicellulases which hydrolyzed approximately 16%, while cellulases

Table 4.9: Effect of incubation of the xylanase enzyme system at 50°C for 1 hour on its activity[a]

Enzyme	*T. reesei*[b]	*T. reesei*[c]	*T. reesei*[d]	Gamanase	Novozym
	Loss of Original Activity (%)				
Arabinogalactanase	<1	<1	<1	<1	<1
Galactomannanase	<1	88	21	2	24
Galactanase	<1	15	<1	13	<1
Xylanase	99	99	89	86	85
Arabinosidase	34	42	13	34	22
Galactosidase	15	17	5	<1	<1
Mannosidase	75	22	<1	11	4
Xylosidase	92	99	<1	40	<1
β-glucanase	4	<1	4	16	4
Glucosidase	20	25	<1	15	-
1:3α Glucanase	57	56	50	65	60
1:3β Glucanase	83	58	87	77	80
1:4β Glucanase	80	50	43	33	38
Cellulase	95	84	84	91	98
Arabinoglucanase	<1	<1	8	2	6

[a]Substrates used in these tests are shown in Table 4.8

[b]*Trichoderma reesei* was cultivated in shake flasks in medium containing canola meal as a carbon source

[c]*Trichoderma reesei* was cultivated in shake flasks in medium containing Solka-floc as a carbon source

[d]*Trichoderma reesei* was cultivated in a fermentor in medium containing Solka-floc as a carbon source

caused about 4%, and β-xylanase about 1% hydrolysis, on a dry weight basis of the substrate. Of these activities, enzyme preparations from *T. reesei* appear to contain most of them except for those required for the hydrolysis of the arabionglucan, the polysaccharides that are released from canola meal as a result of autoclaving. Even the cultivation of *T. reesei* in a medium containing canola meal did not appear to induce the formation of these enzymes.

A pretreatment such as autoclaving followed by enzymatic hydrolysis using *T. reesei* enzyme system or commercially available Gamanase or Novozym preparations appear to cause the solubilization of about 20% of canola meal on a dry weight basis or over 70% of the total polysaccharides present in the meal. These results are important for the utilization of polysaccharides. Since enzyme system produced by *T. reesei* contains very small amounts of proteolytic enzymes (Khan *et al.*, 1989*a*), the removal of these polysaccharides may also be useful in increasing the availability of protein in feeds supplemented with canola meal (Gattinger *et al.*, 1990*b*).

Table 4.10: Hydrolysis of canola meal by untreated and treated enzyme preparations to inactivate cellulase and xylanase activities

Enzyme preparation	Water soluble sugars released (% ww^{-1} of canola meal) Free (1)	Total[a] (2)	Polysaccharides (2)-(1)
T. reesei[b]	13.6	21.5	7.9
T. reesei[bc]	7.6	14.5	6.9
T. reesei[c]	9.9	18.0	8.1
T. reesei[cc]	5.9	12.1	6.2
T. reesei[d]	12.1	20.0	7.9
T. reesei[dc]	7.9	15.8	7.9
Gamanase	15.4	15.4	<1
Gamanase[c]	14.3	14.3	<1
Novozym	18.6	18.6	<1
Novozym[c]	17.4	17.4	<1

[a] After hydrolysis with hydrochloric acid

[b] *Trichoderma reesei* was cultivated in shake flasks in medium containing canola meal as a carbon source

[c] *Trichoderma reesei* was cultivated in shake flasks in medium containing Solka-floc as a carbon source

[d] *Trichoderma reesei* was cultivated in a fermentor in medium containing Solka-floc as a carbon source

[e] Enzyme preparations were incubated at 50°C for 24 h to inactivate cellulase and hemicellulase activities before use in this hydrolysis

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

The main conclusions of the project are outlined below:

1. *Trichoderma reesei* has the ability to grow on canola meal and produce xylanase and some other enzymes which are required for synergistic hydrolysis of complex materials. These enzymes were produced in comparable or higher amounts than that produced from expensive carbon sources such as Solka-floc, cellulose, glucose, lactose, sucrose, or purified xylans.

2. The physicochemical properties such as pH and temperature optima and temperature stability of the enzyme system produced using canola meal are similar to those from the system using the more expensive substrate Solka-floc.

3. The xylanase enzyme system produced using canola meal as a substrate is inhibited by the divalent cations Hg^{2+} and Mn^{2+}.

4. The enzyme system produced using canola meal was more or equally efficient as compared to that produced using Solka-floc in hydrolyzing canola meal, corn cobs, corn and wheat brans, straw, and larchwood xylan to fermentable sugars.

5. With respect ot the enzymatic hydrolysis of canola meal:

   - thermal pretreatment of canola meal is necessary for its maximal hydrolysis; the enzyme preparations hydrolyzed over 20% (w/w) of pretreated canola meal which constitutes over 70% saccharification of the total polysaccharides compared to only 3% (w/w) in the untreated canola meal
   - 10 ml of enzyme preparation produced using canola meal containing 1400 IU of xylanase was necessary for maximal hydrolysis of 1 g
   - a higher extent of saccharification was acheived at 50°C compared to 37°C
   - heat pretreatment of canola meal releases water soluble polysaccharides consisting mainly of arabinose and glucose; these polysaccharides are not hydrolyzed by the *T. reesei* enzyme system but are hydrolyzed by the commercial Gamanase and Novozym enzyme systems
   - the total sugars produced by the canola meal enzyme preparation was higher than that produced by the Gamanase or Novozym preparations

6. The hemicellulases were found to be more stable at 50°C than xylanases or cellulases.

7. Saccharification of canola meal is mainly brought about by hemicellulases which are able to degrade arabinogalactan, arabinoglucan, galactan and galactomannan, while cellulases and xylanases play a minor role.

5.2 Recommendations

The following recommendations are made based on the findings of this work:

1. The results of this work show that xylanase can be produced by *T. reesei* using canola meal as a carbon source in shake flasks. The results also show that xy-

lanase production is greatly affected by culture conditions. The next step is to investigate this process under controlled conditions in a fermentor.

2. Another area which merits further investigation is the use of solid state fermenatation of canola meal using *T. reesei*, since xylanase production was maximized within the rheoligical limits of the liquid fermentation process. The work of Atev (1986), shows that *T. reesei* is capable of growing and developing under solid-state fermentation conditions using various lignocellulosic substrates as inducers of enzyme biosynthesis.

3. The results of this study also showed the pretreatment of canola meal was necessary before its enzymatic saccharification. Further investigations should be carried out to determine different methods of pretreatment, their effectivenesss and cost must also be considered.

4. The results of this study showed that in addition to xylanase, a variety of other enzymes were also produced when canola meal was used as a carbon source. This use of canola meal to enhance production of some or all of these enzymes should also be investigated.

5. The use of the arabinoglucan polymer released as a result of autoclaving canola meal as a commercial specialty substrate for determining arabinoglucan activity should be investigated.

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Appendix A

Standard Curves

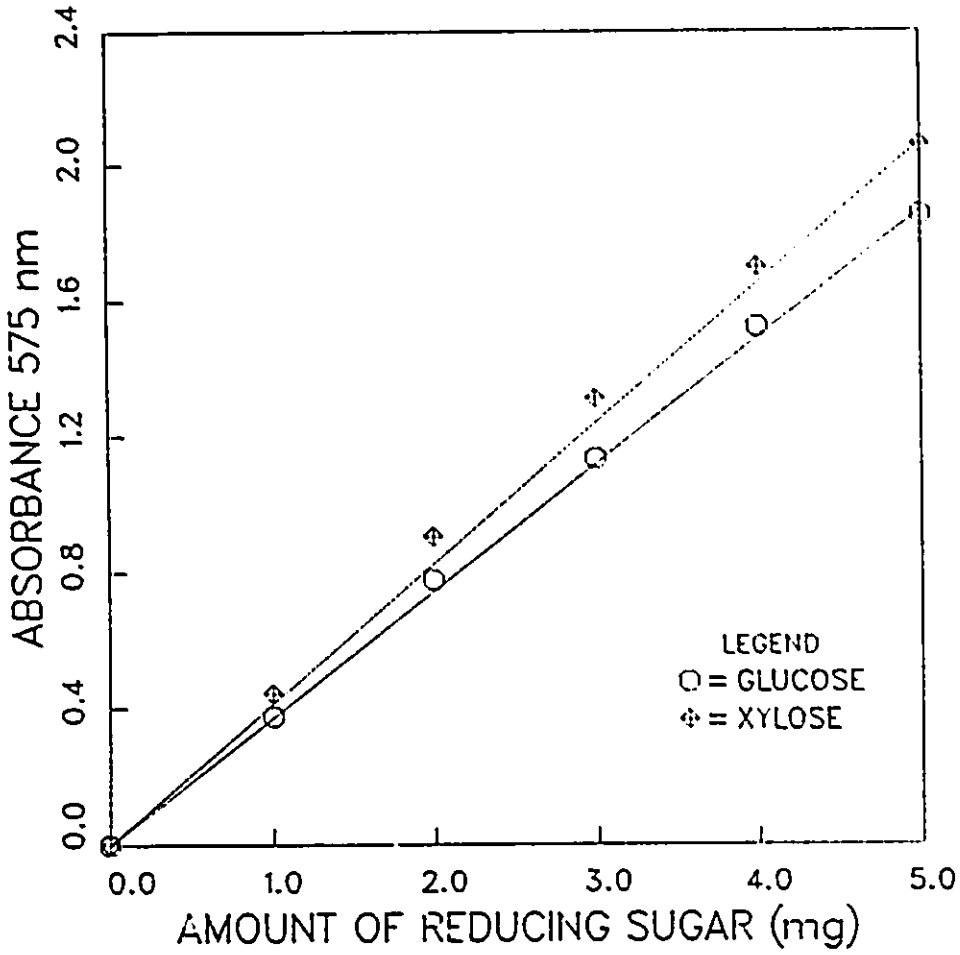

Figure A.1: Standard curve for the measurement of reducing sugars using the dinitrosalicylic acid reagent

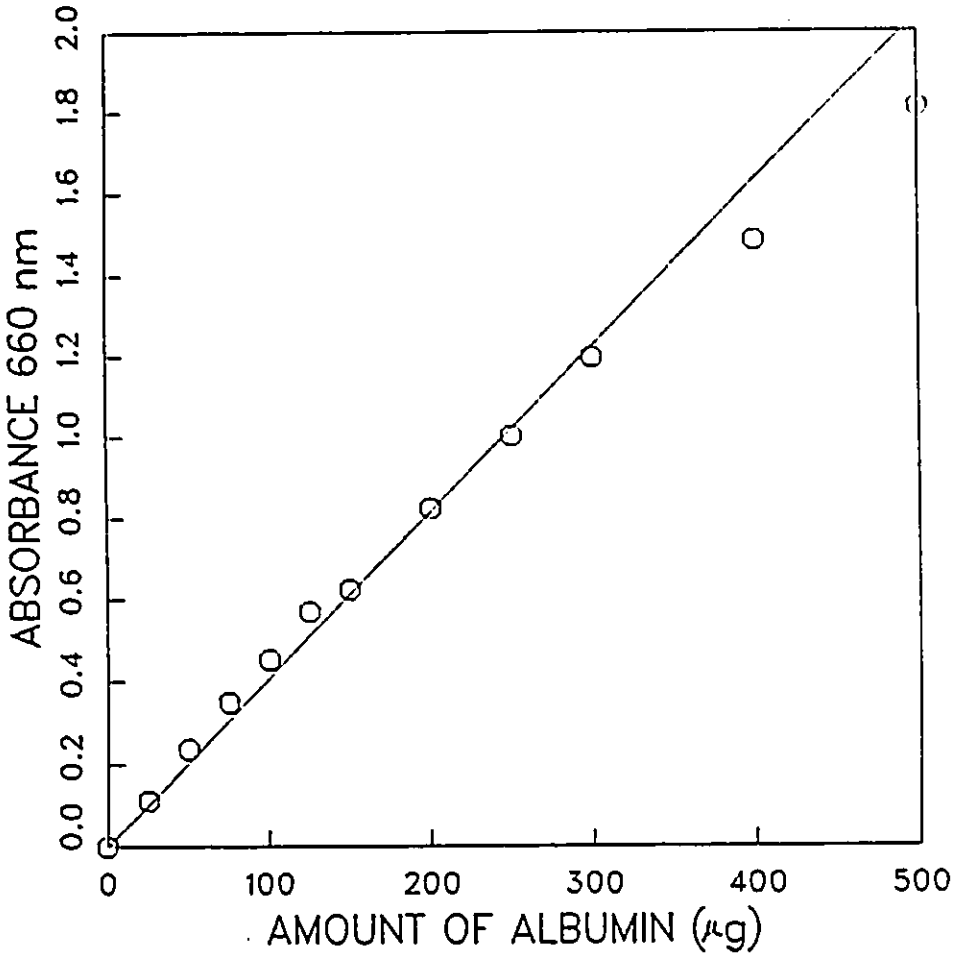

Figure A.2: Standard curve for the measurement of protein and nitrogen content using the Folin-Phenol reagent

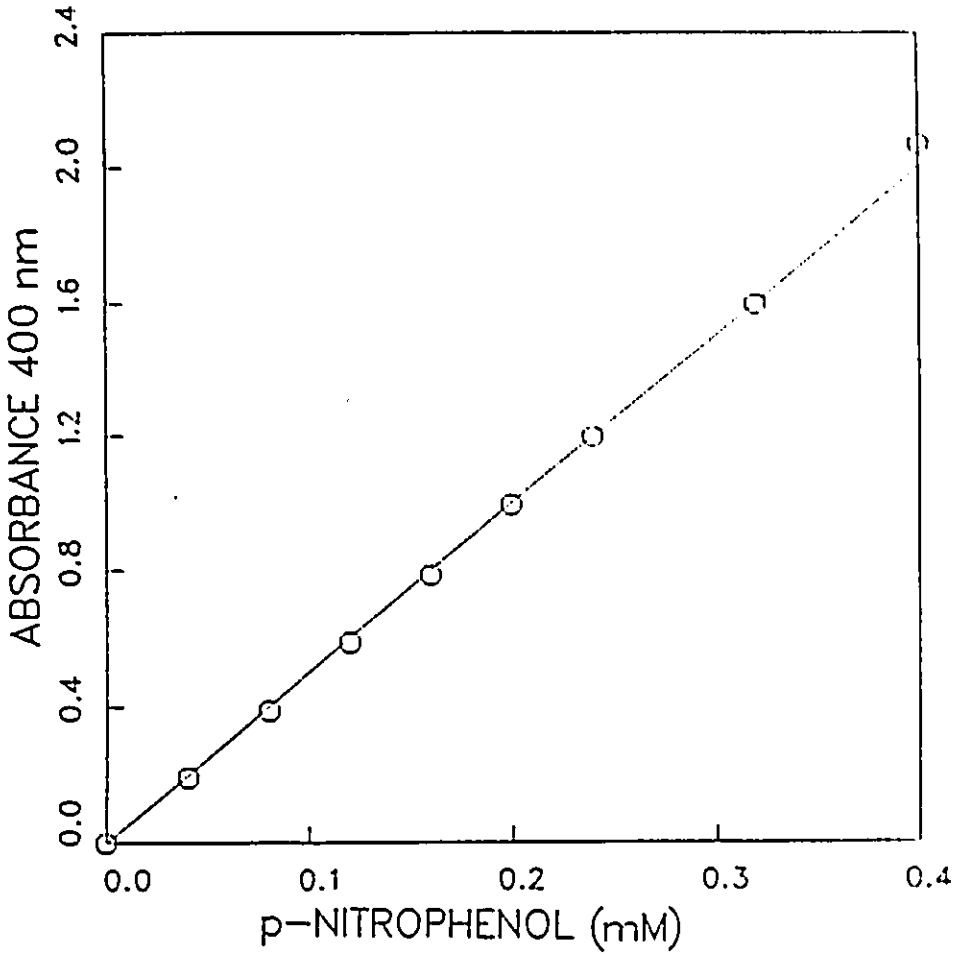

Figure A.3: Standard curve for the measurement of p-nitrophenol for the determination of β-xylosidase activity

Appendix B

Dilution Effects on Enzyme Activity

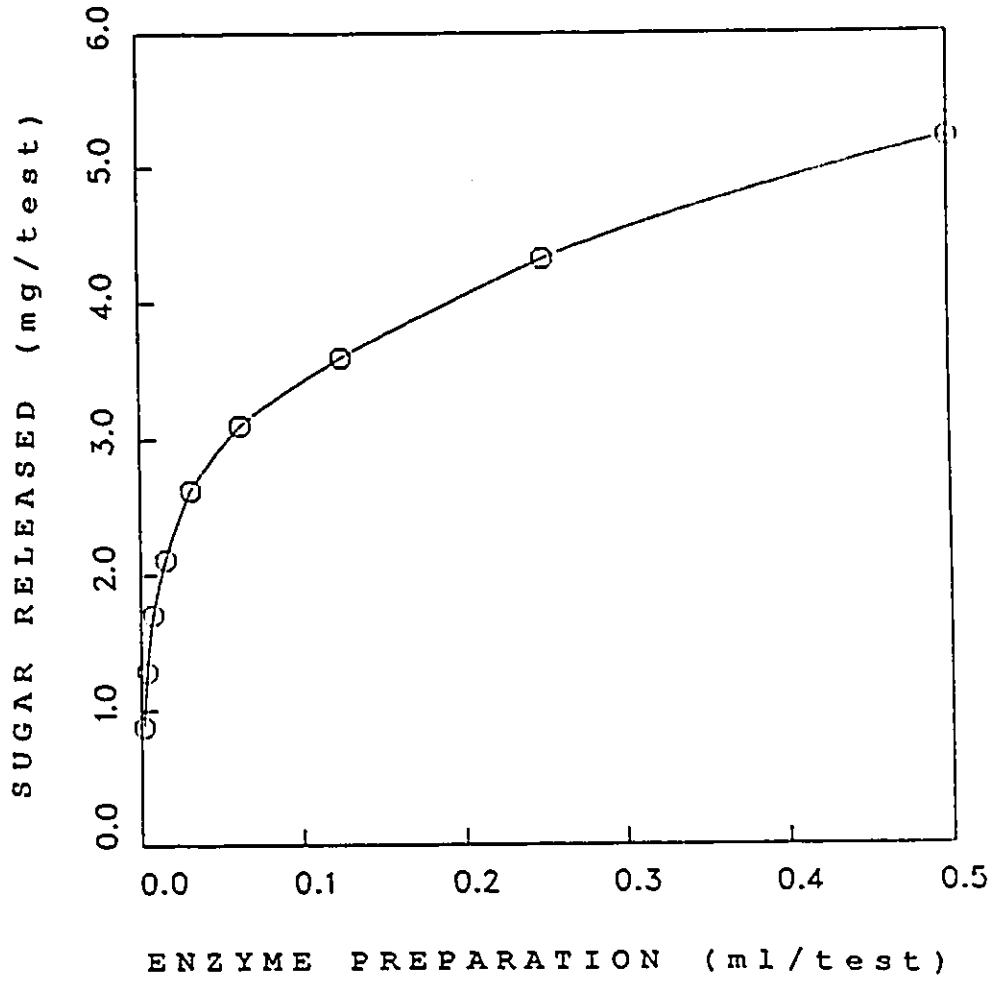

Figure B.1: Effect of enzyme concentration on its activity

Appendix C

Raw Data

Data for Figure 4.1

Time (Days)	Xylanase Activity (IU/ml)					
	2 % Cellulose			2 % Solka-floc		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	0	0	0
3	1	1	1	38	36	37
5	3	3	3	-	-	-
6	4	4	4	81	76	79
7	6	6	6	97	97	97
10	12	12	12	145	145	145
12	11	12	11	140	129	135

Data for Figure 4.2

Time (Days)	Xylanase Activity (IU/ml)								
	1 % Glucose			1 % Lactose			1 % Sucrose		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	0	0	0	0	0	0
3	-	-	-	2	1	2	1	1	1
5	2	2	2	4	3	4	3	3	3
7	9	8	9	4	4	4	4	4	4
9	20	19	20	5	5	5	4	4	4
11	31	31	31	8	8	8	5	4	4
13	36	33	35	8	8	8	4	4	4

Data for Figure 4.3

Time (Days)	Xylanase Activity (IU/ml)								
	1 % Oat Spelts Xylan			1 % Larchwood Xylan			1 % Solka-Floc		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	0	0	0	0	0	0
3	48	51	50	89	64	77	65	76	71
5	65	73	69	89	85	87	163	171	167
7	92	91	92	95	96	96	184	182	183
9	108	119	114	134	117	126	207	207	207
11	92	97	95	116	79	98	164	196	180

Data for Figure 4.4

Time (Days)	Biomass (g/l)								
	1 % Glucose			1 % Lactose			1 % Sucrose		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	0	0	0	0	0	0
3	63	63	63	12	12	12	15	11	13
5	61	61	61	8	8	8	8	8	8
7	53	48	51	9	7	8	12	9	11
9	37	39	38	6	5	6	5	6	6
11	37	34	36	8	4	6	7	6	6
13	33	31	32	7	5	6	6	6	6

Data for Figure 4.5

Time (Days)	Xylanase Activity (IU/ml)								
	0 % Bactopeptone			3.3 % Bactopeptone			10 % Bactopeptone		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	0	0	0	0	0	0
3	13	13	13	21	23	22	9	9	9
6	50	54	52	136	156	146	97	89	93
9	77	89	83	151	159	155	176	190	183
12	73	79	76	148	154	151	171	189	180

Data for Figure 4.6

Carbon (%, w/v)	Solka-floc as Carbon Source								
	Maximum Xylanase Activity (IU/ml)								
	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5	Flask 6	Flask 7	Flask 8	Average
0	0	0	0	0	0	0	0	0	0
0.5	106	113	119	130	-	-	-	-	117
1	203	200	210	195	196	211	215	226	207
1.5	198	176	166	180	-	-	-	-	180
2	142	95	155	160	159	155	130	132	141
3	103	123	-	-	-	-	-	-	113
4	100	116	-	-	-	-	-	-	108

Data for Figure 4.6 (Continued)

Carbon (%, w/v)	Canola Meal as Carbon Source								
	Maximum Xylanase Activity (IU/ml)								
	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5	Flask 6	Flask 7	Flask 8	Average
0	0	0	0	0	0	0	0	0	0
0.5	22	20	-	-	-	-	-	-	21
1	25	37	-	-	-	-	-	-	31
2	48	57	50	57	-	-	-	-	53
3	91	95	-	-	-	-	-	-	93
4	101	123	94	94	99	111	113	97	104
5	137	129	117	136	107	132	142	132	129
6	183	145	157	167	-	-	-	-	163
8	212	207	219	202	-	-	-	-	210

Data for Figure 4.7

Time (Days)	1 % Solka-floc					
	Xylanase Activity (IU/ml)			pH		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	5.63	5.62	5.63
3	85	70	77	6.37	6.19	6.28
5	177	136	155	6.34	6.39	6.37
7	182	144	163	6.42	6.41	6.42
9	212	202	207	6.58	6.56	6.57
11	167	155	161	6.62	6.63	6.63

Data for Figure 4.8

Time (Days)	8 % Canola Meal					
	Xylanase Activity (IU/ml)			pH		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	5.79	5.79	5.79
3	16	13	15	7.26	7.20	7.23
5	95	95	95	7.32	7.24	7.28
7	166	160	163	7.40	7.14	7.27
10	207	205	206	7.35	7.21	7.28
11	195	226	210	7.57	7.10	7.34

Data for Figure 4.9

Time (Days)	Xylanase Activity (IU/ml)					
	Marbles Present			Marbles Absent		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	0	0	0
3	15	12	14	17	24	21
5	46	53	50	69	94	82
8	107	105	106	131	123	127
10	127	120	124	119	134	127
12	154	158	156	154	189	172
15	150	160	155	154	171	163

Data for Figure 4.10

Time (Days)	Xylanase Activity (IU/ml)					
	Tween 80 Present			Tween 80 Absent		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	0	0	0
3	85	70	88	59	55	57
5	177	136	157	75	79	77
7	149	141	145	96	96	96
10	201	191	196	97	109	103
12	187	163	175	98	94	96

Data for Figure 4.11

Time (Days)	Xylanase Activity (IU/ml)					
	Temperature 28°C			Temperature 37°C		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	0	0	0
3	10	12	11	40	33	37
6	77	85	81	120	136	128
9	91	114	102	122	134	128
12	97	92	95	114	124	119

Data for Figure 4.12

Initial pH	Maximum Xylanase Activity (IU/ml)		
	Flask 1	Flask 2	Average
3.61	48	48	48
4.74	87	96	92
5.41	65	67	66
5.73	48	50	49

Data for Figure 4.13

Time (Days)	Xylanase Activity (IU/ml)								
	2.5 % Inoculum			5 % Inoculum			10 % Inoculum		
	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average	Flask 1	Flask 2	Average
0	0	0	0	0	0	0	0	0	0
3	9	17	13	12	17	15	12	12	12
5	34	69	52	81	73	77	78	77	78
7	49	85	67	71	93	82	82	93	88
9	77	121	99	98	129	114	96	98	97
11	61	93	77	76	94	85	94	105	100

Data for Figure 4.14

pH	Maximum Xylanase Activity (IU/ml)					
	Solka-floc Preparation			Canola Meal Preparation		
	Test 1	Test 2	Average	Test 1	Test 2	Average
2.97	17	19	18	14	14	14
4.15	142	172	157	142	134	138
5.13	193	207	200	201	197	199
6.14	190	200	195	199	185	192
7.08	84	90	82	83	79	81
8.00	22	18	20	27	13	20

Data for Figure 4.15

Temperature (°C)	Maximum Xylanase Activity (IU/ml)					
	Solka-floc Preparation			Canola Meal Preparation		
	Test 1	Test 2	Average	Test 1	Test 2	Average
20	13	11	12	11	11	11
30	40	28	34	29	19	24
40	87	73	80	65	55	60
50	197	200	196	139	137	138
55	184	190	187	130	136	133
60	156	168	162	116	124	120
70	30	18	24	16	18	17

Data for Figure 4.16

Time (hours)	Maximum Xylanase Activity (IU/ml)					
	Solka-floc Preparation			Canola Meal Preparation		
	Test 1	Test 2	Average	Test 1	Test 2	Average
0	143	149	146	143	153	148
0.5	165	175	170	146	156	151
1	137	149	143	133	145	139
2	105	117	111	87	93	90
4	76	90	83	53	65	59
6	45	55	50	31	45	38
11	28	38	33	13	19	16
24	10	16	13	5	3	4

Data for Figure 4.17

Time (hours)	Saccharification (%)					
	Solka-floc Preparation			Canola Meal Preparation		
	Test 1	Test 2	Average	Test 1	Test 2	Average
0	0	0	0	0	0	0
1	54.0	55.0	54.5	55.5	-	55.5
1.5	-	58.2	58.2	60.1	-	60.1
2	59.8	59.8	59.8	57.1	57.5	57.3
4	67.0	68.6	67.8	68.0	68.6	68.3
6	69.9	70.7	70.3	70.1	70.5	70.3
8	73.8	74.8	74.3	71.3	74.1	72.7
12	75.6	76.5	76.1	76.0	76.1	76.1
16	-	75.0	75.0	76.8	77.0	76.9
24	76.6	77.0	76.8	76.6	78.3	77.5

Data for Figure 4.18

Time (hours)	Saccharification (%)					
	Solka-floc Preparation			Canola Meal Preparation		
	Test 1	Test 2	Average	Test 1	Test 2	Average
0	0	0	0	0	0	0
1	6.6	7.2	6.9	6.7	-	6.7
2	9.4	9.8	9.6	8.8	9.0	8.9
4	9.9	11.9	10.9	11.2	12.6	11.8
6	11.0	11.6	11.3	12.1	12.9	12.5
8	12.9	13.7	13.3	14.0	15.0	14.5
12	13.1	13.9	13.5	14.2	14.6	14.4
16	14.0	14.4	14.2	15.8	16.6	16.2
24	14.9	15.3	15.1	16.6	16.4	16.5

Data for Figure 4.19

Time (hours)	Saccharification (%)					
	Solka-floc Preparation			Canola Meal Preparation		
	Test 1	Test 2	Average	Test 1	Test 2	Average
0	0	0	0	0	0	0
1	19.9	21.5	20.7	27.3	28.5	27.9
2	21.5	22.1	21.8	31.8	32.4	32.1
4	28.5	28.7	28.6	37.0	37.2	37.1
6	28.4	29.4	28.9	39.9	41.3	41.1
8	29.3	-	29.3	40.2	41.2	40.7
16	33.5	34.3	33.9	43.6	43.6	43.6
24	34.1	34.5	34.3	44.2	45.0	44.6

Data for Figure 4.20

Enzyme Conc. (ml/g)	Saccharification (%,w/w)					
	Solka-floc Preparation			Canola Meal Preparation		
	Test 1	Test 2	Average	Test 1	Test 2	Average
0	0	0	0	0	0	0
2.5	6.4	6.2	6.3	7.7	7.1	7.4
5	8.2	8.2	8.2	8.8	8.9	8.9
10	8.4	9.0	8.7	9.9	10.5	10.2
20	9.1	9.1	9.1	9.7	10.7	10.2

Data for Figure 4.21

Time (hours)	Saccharification (%)					
	Solka-floc Preparation, 37°C			Canola Meal Preparation, 37°C		
	Test 1	Test 2	Average	Test 1	Test 2	Average
0	0	0	0	0	0	0
2	17.0	18.0	17.5	29.0	31.0	30.0
4	20.5	21.7	21.1	33.2	35.4	34.3
8	24.9	25.1	25.0	36.8	39.0	37.9
24	28.0	28.4	28.2	46.6	47.0	46.8

Data for Figure 4.21 (Continued)

Time (hours)	Saccharification (%)					
	Solka-floc Preparation, 50°C			Canola Meal Preparation, 50°C		
	Test 1	Test 2	Average	Test 1	Test 2	Average
0	0	0	0	0	0	0
2	19.5	20.5	20.0	35.9	36.7	36.3
4	25.9	27.7	26.8	35.2	36.2	35.7
8	29.9	29.3	29.6	42.1	42.9	42.5
24	33.0	33.4	33.2	47.6	49.6	48.6