THE UNIVERSITY OF CALGARY

SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

by

EBO BUDU - AMOAKO

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled SIMULTANEOUS SACCHARIFICATION AND FERMENTATION submitted by EBO BUDU-AMOAKO in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

A study was conducted using a crude amylase preparation produced by growing Aspergillus awamori and Aspergillus niger on raw ground corn. The Koji preparation was used to hydrolyze the starch of raw ground corn to sugars during simultaneous fermentation of the sugars to ethanol by distillers yeast in a non-sterile environment.

A fairly comprehensive study was made into the production of the Koji and the simultaneous fermentation of sugars produced by the hydrolysis of the starch to ethanol by yeast leading to the optimization of their production. At least 1.5 units/mL of total amylolytic activity /50 g corn was found to be required to ensure reasonable fermentation efficiencies. Heating and drying Koji at $41^{0}C$ had no detrimental effects on the amylolytic activities. Such Koji could be stored at room temperature with not much change in the amylolytic activities for a period of 120 days.

Corn ground with one-half inch hammermill was found to produce almost as much ethanol as other corn samples further ground to substantially reduce their size.

Comparison was also made of two fermentation technologies (SSF and SBF) with the conventional fermentation technology. These trials indicated that SSF is just as efficient as the conventional process whilst it

is more efficient and less time-consuming than saccharification before fermentation (SBF) using Koji amylases. All the three methods resulted in good theoretical yields, but SSF proved to be superior.

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I.0 INTRODUCTION

1.1 The Need for Alternative Fluid Fuel Resources

Demand for the natural resources of the earth is rising as the number of people also continues to increase. In fact, the demand is rising faster than the population itself increases, largely because people expect more of their society and their environment. Therefore, the strain on world resources continues to grow.

One of the principal natural resources upon which modern society is built is energy. The biggest source of today's energy consists of fossilized products of ancient photosynthetic plants that grew on the earth's surface 100 million years ago or more. The use to which we put these resources are utility generation, residential and commercial heating, industrial processing and transportation. About 95% of our present energy sources are dependent on these ancient photosynthetic deposits (Calvin, 1984).

There is no wisdom in our sole dependence on fossil energy because our future is threatened by the fact that it is not replenishable. In the last 100 years, industrial development has consumed enormous quantities of these fossil energy resources (Mullins, 1985). The amount of oil found per foot of well drilled has drastically dropped over the years. In the United States, which of course reflects the

situation in all oil producing areas, this has dropped from 35 barrels in 1945 to less than 15 in 1978 (Calvin, 1984). Another way of expressing the same idea is to show how much energy you have to expend to find a new barrel of oil or gas to replace the fuel used. The energy expended will reach 6 million BTU, the total amount of energy in a barrel of oil, by about 1990. If the cost of finding and extracting a barrel of oil exceeds the total energy content, it is not economical to search for more, even if there are undiscovered reserves. Industrial society today is strongly dependent on this fuel as its source of energy but it is supplied by a few countries which, to a large extent, have controlled the world's economy during the last decade (Carreira and Ljungdahl, 1983).

There is no need to point out the dimensions of energy predicaments facing the world (especially the developing world). In view of these problems, the need to develop indigenous energy alternatives is both obvious and urgent (Barnard, 1984). It behoves us, therefore, to give some thought as to what we can find in the way of alternative fluid fuel resources. We must seriously think of the possibility of finding other alternative sources of liquid fuel.

Research has indicated that, among a host of liquid fuels, ethanol seems a potential liquid fuel substitute because it has relatively good combustion characteristics in

internal combustion engines (Kirk-Othmer, 1984). The use of ethanol in motor vehicles is neither a new technology nor a new concept. This subject dates back into the "20s" and ethanol (alcohol) has often been used in both war and peace. Henry Ford's Model T was designed so that it would run on alcohol, gasoline or any mixture of these fuels. During World War II, the U.S. operated ethanol plants in Omaha to produce motor fuel for the army, and gas stations in Kansas, Nebraska and Illinois sold the alcohol (ethanol)/gasoline blends - then called Argol. In 1934 Hiram Walker marketed a gasoline/ethanol (alcohol) mixture called Alcoline (Lyons, 1981).

More interest and controversy, however, has been generated in the use of ethanol as fuel for motor vehicles in the 1970's and early 1980's than almost any other renewable energy source because of the oil crisis in the early 1970's (Weller et al., 1984). In recent years, ethanol use as a fuel for motor vehicles has occurred either as a fuel extender in ethanol-gasoline blends (gasohol), or as neat ethanol. Ethanol can serve as a fuel extender without requiring significant engine and fuel system modification or loss of power and efficiency (Kirk-Othmer, 1984), whereas the hydrous form (80-95%), can be used directly after slight engine modification (Lyons, 1981).

1.2 Energy Economics of Ethanol Production

The basic problem with conventional yeast fermentation of carbohydrates and conventional purification to yield fuel-grade ethanol is that more energy can be utilized to manufacture a unit of ethanol than the energy contained in that unit. For example, The Watt Committee report (Rothman et al., 1981) illustrated the basic inefficiency of conventional alcohol (ethanol) production. This report stated that one gallon of ethanol contains 90,000 BTU, yet it requires 139,000 BTU to produce it from grain by conventional fermentation. Another example is illustrated in Table 1.

TABLE 1.Net Energy Production Ratios for Ethanol Production from Corn.

Source	Energy Consumed	Energy as Alcohol	N*
Office of Technology Assessment	320 MJ	128 MJ	-0.69
Amoco Oil Company	46.29 MJ	21.9 MJ	-0.54

*N = Net energy output ratio from(Kirk-Othmer,1984).

The net energy production is negative.

In 1981 the U.S. Department of Energy predicted that, by the mid 1980's, some 3,000 plants would be required to meet their targeted production of ethanol. Today there are

only about 50 - 100 operating plants (Lyons, 1983). Many have either failed to start production or have started up and then closed down because the operating costs are in excess of the potential sale price of ethanol (Lyons, 1983).

Now the emphasis or objective of the production of ethanol is to make it cost effective if it is meant to effectively replace gasoline. Much research has been conducted in the last decade, aimed at improving the ethanol fermentation process in order to reduce production costs.

1.3 Recent Approaches to Improve the Economics of Ethanol Fermentation

Some approaches to improve the economics of ethanol production are in the following areas:

(a) Simultaneous saccharification and fermentation with enzymes and yeast (Ueda et al., 1981; Weller et al., 1984).

(b) Use of packed columns containing live immobilized yeast or both enzymes and yeast cells through which glucose solutions are passed (Dermot, 1979).

(c) Use of bacteria instead of yeast to shorten fermentation times (Rogers et al., 1980).

(d) Use of thermophilic anaerobes for one step hydrolysis and fermentation of biomass (Carreira and Ljungdahl,

1983).

(e) Recombinant genetic approaches for efficient ethanol production (Skotnicki et al., 1982; Tubb, 1984).

(f) Rapid ethanol fermentation using vacuum and cell recycle (Cysewski and Wilke, 1977).

1.4 Conventional Fermentation of Ethanol

The conventional process of fermentation for the conversion of starch mashes to ethanol involves (Fogarty, 1981):

(a) Cooking the starch (gelatinization).

(b) Liquification and saccharification (enzyme hydrolysis).

(c) Alcoholic fermentation by yeasts.

1.5 Enzymes Involved in the Hydrolysis of Starch

In a discussion of enzymes that promote the hydrolysis of starch, it is well to consider briefly some of the structural features of starch which are of particular importance in enzymic reactions. Starch is a high molecular weight polymer of D-glucose. Most starches consist of a mixture of two types of polymers, amylose and amylopectin (Pazur, 1965). The amylose molecule is a long unbranched chain of 300-1,000 α-D-glucopyranose units linked to one

another by α-1-4 glucosidic bonds which give the molecule a tendency to assume a helical conformation. The amylopectin molecule is branched and resembles the animal storage polysaccharide glycogen. It has a branched, tree-like, structure made up of chains of α-1-4 linked α-D glucopyranose units with α-1-6 linkages at the branching points (Goodwin and Mercer, 1982). Although an α-1-3 linkage has been found in amylopectin from waxy maize (Wolfrom and Thompson, 1955), the presence of such a linkage in other types of amylopectin has not been demonstrated.

There are three major groups of enzymes capable of catalyzing the hydrolysis of starch; alpha-amylases, beta-amylases and glucoamylases. Alpha-amylases promote a more or less random fragmentation of the starch molecule by hydrolyzing endo α-D-(1-4) glucosidic bonds in the inner and outer chains of the compound. That considerable amounts of low molecular weight reducing sugars are produced in the initial stages of hydrolysis of starch by these enzymes was indicated in early work (Freeman and Hopkins, 1936). With the advent of paper chromatography, this observation has been confirmed and the reducing sugars have been identified as maltose, maltotriose and maltotetraose (Pazur et al., 1950; Giri et al., 1953). The alpha-amylases cannot hydrolyze the α-D-(1-6) bonds of amylopectin (Pazur et al., 1950), but can bypass them. As a result, starch fragments which are resistant to hydrolysis will be produced from

those portions of the molecule containing the ∝-D-(1-6) linkage. These fragments have been called limit dextrins (Pazur and Ando, 1959), and contain ∝-D-(1-6) bonds as well as some ∝-D-(1-4) bonds. The alpha-amylase limit dextrins are relatively low molecular weight compounds.

In its action on starch, it has been recognized for many years that beta-amylase begins at the non-reducing end of the outer chains and proceeds by stepwise removal of maltose units. Amylose with an even number of D-glucose units is converted completely to maltose while amylose with an odd number of D-glucose units is converted to maltose and a maltotriose. At high concentrations of enzyme and prolonged incubation, maltotriose can be slowly hydrolyzed to D-glucose and maltose (Englard and Singer, 1950). Amylopectin is hydrolyzed like amylose beginning at the non-reducing end of the outer chains. However, since beta-amylase cannot hydrolyze or bypass an ∝-D-(1-6) bond, high molecular weight limit dextrins containing all original ∝-D-(1-6) linkages are produced (Meyer, 1952). A large portion of the amylopectin molecule is therefore, not hydrolyzed by the beta-amylase.

Glucoamylases hydrolyze starch directly to D-glucose by a stepwise removal of glucose from the non-reducing end. They are produced by several species of fungi of the Aspergillus (Kitahara and Kurushima, 1950; Pool and Underkofler, 1953) and Rhizopus groups (Phillips and

Cadwell, 1951), and by certain yeast and bacteria. These enzymes hydrolyze amylopectin, amylose and maltooligosaccharides completely to D-glucose (Pazur and Ando, 1959). Isomaltose (Pazur and Ando, 1960) and nigerose (Pazur and Kleppe, 1962) are also hydrolyzed, demonstrating that the enzyme is capable of hydrolyzing α-D-(1-6) and α-D-(1-3) bonds as well as α-D-(1-4) bonds.

1.6 Gelatinization of Starch

If a suspension of starch in water is heated, the water penetrates the outer layers of the granules and it begins to swell. This occurs as the temperature rises from 60^0C to 85^0C, the granules burst or rupture to elute the starch into the surrounding aqueous phase to produce a viscous paste (Leach, 1965).

The saccharification and distillation processes consume large amounts of energy. Cooking to gelatinize the starch prior to enzymatic hydrolysis consumes approximately 17% of this energy (Weller et al., 1984) and is a major part of the large energy demand that makes the economic feasibility of biological ethanol production seem an unfavourable alternative.

1.7 Raw Starch Hydrolysis and Fermentation

Efforts to reduce the energy costs in ethanol production date back to the late nineteenth century, when starch was just beginning to be defined as to its components: dextrins and glucose. Methods for hydrolyzing raw starch were being studied at that time. In one method, malt extract was added to ungelatinized triturated corn and wheat to produce 2 to 25% glucose and 10 to 14% dextrins within 20 hours (Brown and Heron, 1879). Blish and co-workers (1937) published their observation of the actions of wheat amylases on raw wheat starch; they claimed normal wheat flour contained an enzyme factor, not alpha-amylase, that accelerated the saccharification of raw untreated wheat starch. Support for this observation was obtained by others (Sandstedt and Gates, 1954; Walker and Hope, 1963).

Balls and Schwimmer (1944), reported that ungelatinized corn, wheat and potato starches could be completely hydrolyzed to glucose by a mixture of extracts of hog pancreas and Aspergillus oryzae Koji grown on wheat bran. Schwimmer (1945) noted that pancreas and Aspergillus are both known to contain α-amylase, but the action of α-amylase alone on uncooked starch, except in the presence of extremely high concentrations of enzyme, reaches completion very slowly. β-amylase on the other hand had been shown by other experimenters (Kneen et al., 1941; Lu and Ma, 1943),

to have no effect on raw starch. This was also observed by Schwimmer who used β-amylase as a single enzyme and noticed it had no action alone on raw starch, and no apparent effect on the action of the pancreas - mold mixture. He postulated that the Koji contained a complementary factor that caused the complete and rapid digestion of raw starch. After observing that maltase added to a preparation of α-amylase increased the digestion of raw starch, he suggested that this factor may be an α-glucosidase such as maltase. By using a preparation (from pancreas) containing α-amylase but practically no maltase, and a preparation (from Aspergillus) containing maltase but no α-amylase, he concluded that the rapid and complete digestion of raw starch occurs because of the removal of maltose. He conceded, however, that there was the possibility that other enzymes not taken into account may exist in the partly purified preparation from pancreas and Aspergillus.

This so-called synergistic effect of maltase has been discussed under such terminology as "amylase activation" (Pringsheim and Loew, 1932), and "complementary action" (Weidenhagen and Wolf, 1931). In 1948, Corman and Langlykke added to the understanding of the Koji amylases when they discovered a fraction of the amylases (later named glucoamylases) which was highly glucogenic. This might have been the helper factor that Balls and Schwimmer encountered in their experiments. Further studies of Koji amylases by

Yamasaki and Ueda (1951), revealed that black-Koji (A. niger and A. awamori) amylases had stronger raw starch hydrolyzing activity than those of yellow-Koji (A. oryzae) and malt amylases at an optimum pH of 3.6. Ueda (1958) then reported that the Koji amylases from A. awamori contained two fractions, one which had raw starch digestive activity and the other that did not.

Glucoamylases in Koji amylases of both A. niger and A. awamori were again shown to be of two fractions (Pazur and Ando, 1959; Watanabe and Fukimbara, 1965). Smiley et al., (1971) tried to determine the reason for the existence of these two fractions but were unable to come to any conclusion. Ueda et al., (1974), later confirmed the presence of the two glucoamylases. They reported one of the glucoamylase fractions was highly active in raw starch digestion which likely had given the Koji amylase fraction he discovered in 1958 its raw starch degrading portion and referred to it as glucoamylase I (had a strong debranching activity and is highly active in raw corn starch digestion) and the other with weak degradation activity as glucoamylase II (had weak debranching activity and is weakly active in raw corn starch digestion).

It is puzzling as to how starch molecules held in the form of a granule (raw starch) could be that susceptible to the hydrolytic enzymes. The dissolving action of various amylases on raw granular starches was studied by Leach and

Schnoch (1961), and it was suggested that starches may have a porous granular structure that is accessible to the enzyme(s). This has since been demonstrated by electron microscopy (Leach, 1965).

Based on the discovery of raw starch degrading capability, the use of raw starch and Koji enzymes has stimulated research recently with the aim of reducing energy costs in biological ethanol production. One of the most progressive is Ueda, who has shown that a dual culture of Aspergillus awamori and A. niger grown on sterile wheat bran produces glucoamylases that hydrolyze raw corn starch (Ueda and Koba, 1980). He has also shown that this hydrolysis of raw starch can be carried out simultaneously with a yeast fermentation of the sugars to ethanol in one reaction vessel. He also developed a continuous fermentation system that used Aspergillus glucoamylase and Pseudomonas isoamylase to hydrolyze raw corn starch. This system produced 7% (v/v) ethanol in three days, but required periodic removal of inhibitory substances. Additionally Ueda et al. (1981), developed a simultaneous hydrolysis and fermentation process for raw ground cassava. Further studies reported 89.0 to 90.0% theoretical ethanol yields from both raw corn and cassava starch (Park and Rivera, 1982). A simultaneous hydrolysis and fermentation of raw ground corn has also been developed by Matsumoto et al. (1982). They reported the first successful industrial scale

ethanol fermentation from corn with a non-cooking system. Their process used amylases from Rhizopus for hydrolysis. They reported 88.5% theoretical ethanol concentrations of 14.5% (v/v) in the beer. A recent ethanol fermentation from raw corn by aspergilli hydrolysis with concurrent yeast fermentation has been developed by Weller et al. (1984). They used crude amylase preparations produced by growing Aspergillus awamori and A. niger on raw ground whole corn. These Koji preparations were used to hydrolyze the starch of raw ground whole corn to sugar during the simultaneous fermentation of the sugar to ethanol by distillers active dried yeast. Their process yielded an average of 89.6% theoretical yield compared to control conventional fermentations that had an average of 89.9% after 72 hours.

Abouzied and Reddy (1986) investigated the utilization of starch by monocultures of different Apergillus species and Saccharomyces cerevisiae and their results showed that starch utilization, amylolytic activity and ethanol yields were low in monocultures of A. niger, A. foetidus, and A. awamori, whereas in cocultures of these organisms with S. cerevisiae there was a dramatic increase in amylolytic activity, starch utilization and ethanol production. They attributed the incomplete utilization of starch by monocultures of Aspergillus species to lack of enough oxygen or to feedback inhibition of amylase activity by glucose released from starch hydrolysis, or both.

Using a synergistic coculture of an amylolytic yeast (Saccharomycopsis fibuligera) and S. cerevisiae, a non-amylolytic yeast Abouzied and Reddy (1987) again fermented unhydrolysed starch to ethanol with conversion efficiencies of 90% of the theoretical maximum.

More recently Kim et al. (1988), have genetically engineered a yeast strain which secretes both ∝-amylase and glucoamylase by transforming a S. diastaticus derivative which originally secretes glucoamylase with the mouse salivary ∝-amylase plasmid pMS12. With this yeast, 97% degradation and 93% utilization of Linter starch was obtained.

1.8 Objectives

The overall objective was to study the feasibility of producing ethanol commercially by a process which eliminated cooking (gelatinization) using a process of simultaneous saccharification and fermentation (SSF).

An attempt was made to optimise the following parameters:

1. production of amylolytic enzymes (Koji)
2. yeast inoculum
3. effect of quantity of fermentable substrate (raw ground corn)
4. fermentation conditions

The result of these experiments were compared to conventional fermentation in assessing process feasibility and possible commercial applicability.

2.0 MATERIALS

2.1 Starchy Products

Raw yellow No. 2 corn ground with a one-half inch. hammermill was obtained from the Alberta Distillers Company. A proximate analysis estimated the starch content as 71.3% of dry weight.

Soluble potato starch was obtained from J.T. Baker Chemical Co.

2.2 Fungal Strains

Aspergillus niger (ATCC 10864) and Aspergillus awamori (ATCC 22342) were obtained from America Type Culture Collection (ATCC).

Saccharomyces cerevisiae, Y-174 was a generous gift of Alberta Distillers Company.

The aspergilli (spores), were maintained on Czapek Dox Agar in 250 mL flasks. Saccharomyces cerevisiae was maintained on culture slants of malt extract agar.

2.3 Commercial Enzymes

The commercial enzymes: Diazyme L-200 (amyloglucosidase) and Termamyl 120 L (α-amylase) were gifts of Miles Laboratories Inc. and Novo Laboratories Inc. respectively.

2.4 Chemicals

Standard reagent grade chemicals were used unless otherwise specified. Bacteriological media were from Difco.

2.5 Media

2.5.1 *Aspergillus* Growth Medium

The growth medium for *Aspergillus* is shown in Table 2.

2.5.2 Yeast Growth Medium

The complex medium of Aiba *et al*. (1968) was employed. The composition is presented in Table 3. Glucose was used as the substrate. Salt and yeast extract were supplied in excess so as not to limit growth, with the phosphate also acting as the buffer.

TABLE 2. Growth Medium for Aspergilli (Czapek Dox Agar)

Component	Concentration (g/L Distilled Water)
Saccharose, Difco	30
Sodium nitrate	3
Dipotassium sulfate	1
Magnesium sulfate	0.5
Potassium chloride	0.5
Ferrous sulfate	0.01
Agar	15

The sucrose portion was made up to 250 mL whilst the agar/salt portion was made up to 750 mL. These two portions were autoclaved separately and once autoclaved they were aseptically mixed.

TABLE 3. Yeast Growth Medium

Component	Concentration (g/L)
Glucose Portion:	
Glucose (Dextrose)	100
Made up to 1/2 final volume with distilled deionized water.	
Salt Portion:	
KH_2PO_4	6
$(NH_4)_2SO_4$	2
$MgSO_4 \cdot 7H_2O$	0.4
Yeast Extract (Difco)	2
pH 6.5	
Salt portion also was made double strength in tap water.	

The glucose and salt/yeast extract portions of the medium were prepared and autoclaved separately at $121^{0}C$ for 15 minutes to prevent caramelization of sugar. Each of the two portions was made up to 1/2 the final volume. The glucose portion was prepared using deionized distilled water only, while the salt/yeast extract portion was prepared simply with tap water. Once autoclaved, these separate portions were aseptically mixed.

2.5.3 Yeast Maintenance Medium

The yeast maintenance medium was as below:

Malt Extract Agar:

Malt extract	2 g
Bacto agar	1 g
Distilled Water	100 mL

This was autoclaved at 15 psi for 20 minutes.

3.0 METHODS

3.1 Determination of Amylolytic Activity

Total amylolytic activity was determined by measuring the amount of reducing sugars produced using Nelson's method (1944).

The assay followed a modification of the method of Ueda and Kano (1975). The assay mixture (7 mL) contained 5 mL of 1.0% boiled soluble potato starch, 1 mL of 0.1 M sodium citrate-HCl buffer pH 5.0 and 1 mL of diluted crude enzyme extract. After incubation for 15 minutes at 30^0C the incubation mixture was placed in boiling water for 10 minutes to stop the enzymatic reaction. One mL aliquots were analysed by Nelson's method.

A unit of activity is defined as the μmoles of reducing sugar produced per mL of enzyme in one minute. The reaction was linear up to 6 units of enzyme.

3.2 Glucoamylase Activity

Glucoamylase (AMG) activity was determined by measuring the release of glucose from the boiled soluble starch using a modification of the method of Ueda and Kano (1975). Twenty μL of the amylolytic reaction mixture (3.1) was used to determine activity.

A unit of glucoamylase is defined as the μmoles of glucose produced per mL of enzyme in one minute.

3.2.1 Quantitative Enzymatic Determination of Glucose

The quantitative enzymatic determination of glucose was done using a modification of the Statzyme

Glucostat 50 (Worthington) assay.

3.2.1.1 Reagent

Statzyme Glucose 50 reagent vial was reconstituted with 50 mL of distilled water and swirled gently to dissolve the contents.

3.2.1.2 Conditions

Time of reaction:	10 minutes
Sample volume:	0.02 mL
Reagent volume:	3.00 mL
Reaction volume:	3.02 mL
Temperature of reaction:	$37^{0}C$

3.2.1.3 Assay

Standard glucose solutions of known concentrations were made. Three mL of reconstituted reagent (Statzyme Glucostat 50) was placed into a small test tube. Twenty µL of distilled water was added. An additional 2 mL of distilled water was again added and this was used to zero the colourimeter.

Three mL each of reconstituted reagent were placed in small test tubes, 0.02 mL each of the known glucose

standards was added to the test tubes, mixed and incubated at 37^0C for 10 minutes. Two mL of distilled water was added to each and the absorbances read and recorded at 500 nm.

A graph of the absorbance at 500 nm versus glucose concentration was plotted. The reaction was linear up to 7 units of enzyme.

3.3 ∝-amylase Activity

∝-amylase activity in the incubation mixture was determined by subtracting glucose (amyloglucosidase activity) from the total amylolytic activity (3.2 and 3.1).

A unit of ∝-amylase is defined as the μmoles of reducing sugars (excluding glucose) produced per mL of enzyme in one minute.

3.4 Production of Extracellular Amylolytic Enzymes by Aspergilli (Koji)

Unless otherwise stated crude amylases used for hydrolysis (saccharification) were produced by a dual culture Koji of Aspergillus niger (ATCC 10864) and Aspergillus awamori(ATCC 22342) grown on ground corn (corn unless otherwise specified).

Fifteen grams of corn was inoculated with 0.1% Triton X-100 suspension containing about 10^8 spores of A. awamori

and A. niger in 10 mL total volume. The spore suspensions were mixed with the corn which was spread in thin layers (about 5 mm) in Petri plates. These plates were then tightly covered with aluminum foil to retain moisture and incubated for 72 hours at 30^0C in a controlled temperature chamber (fresh Koji).

3.4.1 Dehydrated Koji

The dehydrated Koji was initially prepared as the fresh Koji. Plates of fresh Koji were ground in a mortar, well mixed and distributed as thin layers in open vessels. These were put into a vacuum oven for 18 hours at 41^0C under a vacuum of 15 pounds.

After drying the dry (dehydrated) Koji was stored in covered vessels in the dark at ambient temperature.

3.5 Extraction of Amylolytic Enzymes

Each plate of Koji was ground in a mortar with about 100 mL of tap water and the pH adjusted to 4.0 to 4.2. The volume was then made up to 200 mL with tap water. This was filtered through Whatman No. 1 filter paper. The filtrate was assayed for amylolytic enzyme activities.

The amylolytic enzymes in the dried Koji was similarly extracted with a volume of tap water that was proportional

to the aliquot of the dried Koji (200 mL of tap water per 15 g corn).

3.6 Determination of Moisture Content of Different Sieve Mesh Sizes of Corn

The percentage moisture content was determined using a modification of the method of Hall et al. (1970).

Corn ground with one-half inch hammermill (corn) was used as the control. This was further ground in separate batches in a mortar to pass through different sieve mesh sizes. The corn was ground so the total batch passed through the respective sieve mesh.

The mesh sizes of sieve used were:

No. 10 (<2 mm)

No. 18 (<1 mm)

No. 35 (<0.5 mm)

No. 60 (<0.25 mm)

Small clean pans were weighed and about 1 g of the respective corn sizes were put into the pans in triplicate. They were then weighed.

These were placed in a vacuum oven at a temperature of more than 100^0C (114^0C) at 15 pounds of vacuum for 12 hours. They were then left to cool to room temperature in vacuo, then quickly reweighed.

The averages of the loss in weight for the various corn

samples were taken as the moisture contents and the percentages computed.

3.7 Ethanol Assay

A gas chromatographic technique similar to that of Lie et al. (1970) was employed to determine the concentration of ethanol. The salient procedures and conditions involved are outlined below:

3.7.1 Gas Chromatography

Chromatograph:

Shimadzu Gas Chromatograph G C-9A equipped with a FID detector.

Shimadzu Chromatopac C-R2AX.

Column of 2 foot X 1/8 in. Porapak Q (100 to 120 mesh).

Conditions:

Flow Rate

N_2 (Carrier):		50 mL/min
H_2	:	50 mL/min
Air	:	50 mL/min

Temperature

Injection	:	200^0C
Column	:	isothermal at 120^0C

3.7.2 Preparation of Standards

Standard ethanol solutions were periodically prepared in concentrations of 9.50%, 4.75% and 3.16% using absolute ethanol. These three different concentration solutions were employed to construct a standard curve.

Acetone was used as the internal standard for ethanol determination. Standard acetone solutions were periodically prepared at a concentration of 15%.

3.7.3 Sampling

In all cases 500 µL of either standard or sample was mixed with 500 µL of standard acetone solution, and 0.50 µL of this mixture was subsequently injected.

3.7.4 Data Treatment

Concentrations of ethanol were determined by comparing area ratios to standard curves.

3.8 Preparation of Yeast Inoculum

Yeast was inoculated in yeast culture medium in aerobic flasks and incubated at 30^0C at 150 rpm on a shaker for 48 hours.

The required volume of yeast was then taken out, centrifuged at 12,000 rpm and the supernatant discarded. The yeast pellet was then resuspended in the same volume of tap water and the proper volume used to inoculate the fermentation.

3.9 Simultaneous Saccharification and Fermentation

All fermentations were carried out non-aseptically in 250 mL Erlenmeyer flasks (about 65 mm diameter X 85 mm height) in duplicate. A total volume of 200 mL contained, unless otherwise stated, the following:

corn

Koji (fresh or dry)

tap water

fresh aerobically grown resuspended yeast

pH 4.0 - 4.2 (with H_3PO_4).

The fermentations contained magnetic stirring bars and were stirred on magnetic stirrers at a minimal rate required to keep the larger corn particles in suspension. The tops of the fermentors were covered loosely with aluminum foil and all fermentations carried out in a 30^0C incubation room on magnetic stirrers.

3.10 Conventional Fermentation

The conventional fermentation using heat to gelatinize the starch followed a modification of that used by Weller et al. (1984).

Initially, 45 g corn, 85 mL of tap water, 1 mL Termamyl 120 (∝-amylase), and one drop of concentrated ammonium hydroxide were mixed in each of two 250 mL beakers. The contents of the beakers were heated to 85^{0}C and held for 2 hours with constant stirring on magnetic stirrers. They were then cooled to 60^{0}C by adding 45 mL of tap water to each of the beakers.

Next the mash pH's were adjusted to 4.0 to 4.2 with 85% phosphoric acid and 2 mL of Diazyme L-200 (amyloglucosidase) were added to each. The mashes were held at 60^{0}C with constant stirring for one hour on magnetic stirrers before cooling to 35^{0}C by adding 45 mL, each, of tap water and bathing the beakers in tap water.

The mash pH's were again adjusted to 4.0 to 4.2 with 85% phosphoric acid before adding 5 mL of yeast to each of them.

The volumes were each then brought up to 200 mL . A magnetic stirring bar was then added to each to provide stirring during the incubation period and they were covered with aluminum foil. They were incubated for 72 hours at 30^{0}C.

3.11 Saccharification Before Fermentation

A plate of Koji (3.4) was put into each of two 250 mL beakers. Forty five grams of corn and about 150 mL tap water was added to each and the pH's adjusted to 4.0 to 4.2. The volumes were each brought up to 195 mL with tap water, magnetic stirring bars were added and the vessels were covered with aluminium foil. These were incubated at 30^0C for 72 hours. Five mL yeast (3.8) was then added to each and reincubated for an additional 72 hours at 30^0C on magnetic stirrers.

4.0 RESULTS

4.1 Optimization of the Production of Crude Extracellular Amylases by Aspergilli (Koji)

4.1.1 Effect of Time on the Production of Amylolytic Enzymes

Twelve plates of Koji were made (3.4) and after every twenty-four hours amylolytic enzymes were extracted from two plates (3.5) and the total amylolytic activity and amyloglucosidase were assayed.

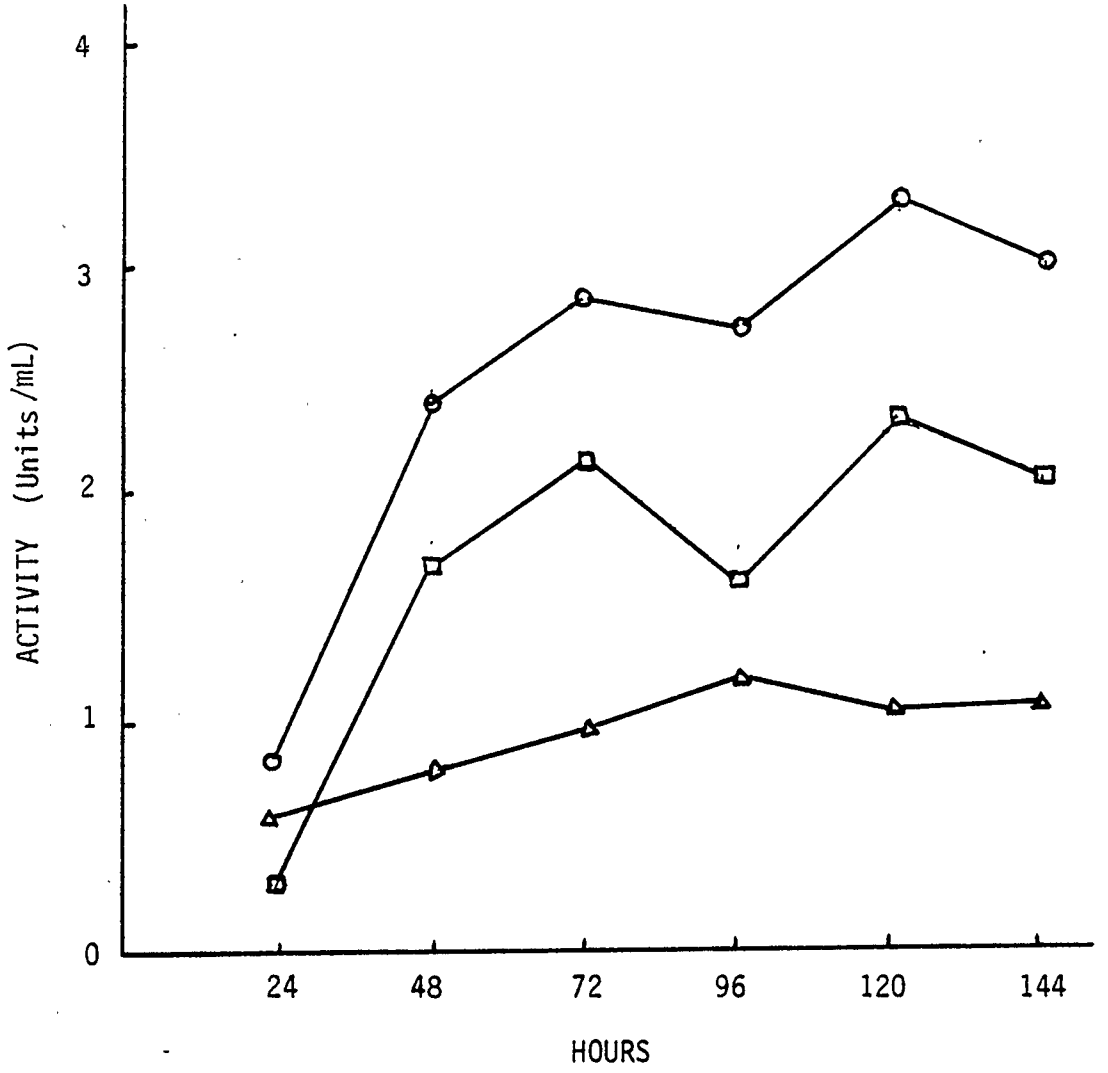
The averages of the effect of time on the production of amylolytic enzymes is shown in Figure 1. The total amylolytic activity increased from 24 hours to 72 hours. From 72 hours to 144 hours the total amylolytic

FIGURE 1 Activity of Amylolytic Enzymes with Time

○ = **Total Amylolytic Activity**

□ = **∝-amylase Activity**

△ = **AMG Activity**


4
3
2
1
0
ACTIVITY (Units/mL)
24
48
72
96
120
144
HOURS

activity remained relatively constant. The ∝-amylase activity also increased from 24 hours to 72 hours then also remained relatively constant. The amyloglucosidase (AMG) activity increased steadily from 24 hours to 96 hours then remained relatively constant.

4.1.2 Effect of the Amount of Corn on the Production of Amylolytic Enzymes

About 10^8 spores, each, of A. niger and A. awamori were inoculated into corn having the following weights in pyrex plates: 5 g, 10 g and 15 g. The amounts of moisture used to inoculate the corn were proportional to the weights of corn used. That is, for 5 g of corn the spores were suspended in 3.3 mL of 0.1% Triton solution; 6.7 mL for the 10 g of corn and 10 mL for the 15 g of corn.

Extraction of enzymes (3.5) was done with 200 mL of tap water (pH 4.2) in each case and amylolytic activities were assayed. The results of the effect of the amount of corn on the production of amylolytic enzymes from averaging duplicate samples is shown in Table 4.

The total amylolytic activity, ∝-amylase and AMG activities in the case of the 5 g corn were lower than that of 10 g and 15 g corn which were almost equal. The ratios of ∝-amylase to AMG in all the three cases were however about the same.

TABLE 4. Amount of Corn and the Activity of Amylolytic Enzymes

Weight of Corn (g)	Total Amylolytic Activity (Units*)	∝-amylase Activity (Units*)	AMG Activity (Units*)
5	0.693	0.518	0.175
10	2.08	1.56	0.52
15	2.13	1.52	0.61

*Units/mL

4.1.3 Effect of Spore Inoculum Size on the Production of Amylolytic Enzymes

Duplicate 15 g corn quantities were inoculated with varying spore numbers of A. niger and A. awamori. The inocula sizes used were as follows: 10^6, 10^7, 10^8 and 5 X 10^8 spores, each, of A. niger and A. awamori with a total volume of 10 mL 0.1% Triton X-100.

These were incubated as before (3.4), enzymes extracted (3.5) and amylolytic activities assayed. The results of this investigation is presented in Table 5. Apart from the spore inoculum size of 10^6 spores, all the other inocula sizes have about the same activities.

4.1.4 Effect of Particle Size of Corn on the Production of Amylolytic Enzymes

Corn (ground with one-half inch hammermill) was used as the control. This was further ground in separate batches in a mortar to pass through different sieve mesh sizes. The corn was ground so the total batch passed through the respective sieve mesh.

The mesh sizes of sieve used were:

i. No. 10 (<2.0 mm)

ii. No. 18 (<1.0 mm)

iii. No. 35 (<0.5 mm)

iv. No. 60 (<0.25 mm)

TABLE 5. Size of Spore Inoculum and Amylolytic Activity

Spore Inoculum Size	Total Amylolytic Activity (Units*)	∝-amylase Activity (Units*)	AMG Activity (Units*)
10^6	0.44	0.029	0.411
10^7	1.65	0.89	0.76
10^8	1.80	1.09	0.71
5×10^8	1.88	1.22	0.66

*Units/mL

Standard Koji (3.4) (15 g corn and 10^8 spores, each, of A. niger and A. awamori) was made with each of the corn particle sizes in duplicate then the enzymes were extracted (3.5) and the amylolytic activities assayed.

The effect that varying the ground corn particle size had on the production of amylolytic enzymes is shown in Table 6. The corn particle sizes from the control to the No. 18 mesh size had about the same amount of activity, being a little lower with respect to the No. 10 mesh size. The amylolytic activity increased with the No. 35 and 60 sizes, almost doubling with the No. 60 in comparison with the activities of that from the control to the No. 18 mesh size.

4.1.5 Effect of Moisture Content During Koji Production of Amylolytic Enzymes

Five batches of Koji were made in duplicate as usual (3.4). However the moisture content, keeping a constant volume of spores in Triton X-100, was varied. All batches contained 5 mL Triton X-100-spore suspension and the additional volume was made up with distilled-deionized water.

The amylolytic enzymes were extracted as before (3.5) from each batch and the total amylolytic activity assayed. The average of the effect of the moisture content

TABLE 6. Particle Size of Ground Corn and Production of Amylolytic Enzymes

Corn Mesh (Size)	Total Amylolytic Activity (Units*)	∝-amylase Activity (Units*)	AMG Activity (Units*)
Control	1.69	1.05	0.64
10	1.36	0.87	0.49
18	1.80	1.11	0.69
35	2.80	2.14	0.66
60	3.50	2.54	0.96

*Units/mL

in the various Koji on the production of total amylolytic enzymes is illustrated in Table 7.

4.1.6 Determination of the Stability of Amylolytic Enzymes in Koji Over 30 Days

One plate of Koji was made using 10^8 of each spore type in 10 mL Triton X-100 and 15 g corn (3.4). This was ground in a mortar and equally divided by weight into four 100 mL beakers.

Fifty mL of tap water (pH 4.0 - 4.2) was used to extract the amylolytic enzymes in one beaker and the activity of enzymes assayed. The other three were heated and dried at 41^0C (under vacuum) for 18 hours. They were cooled, covered tightly with aluminium foil and stored at room temperature in the dark.

Enzymes were extracted from one each of these with 50 mL of tap water (pH 4.0 - 4.2) at 10 days, 20 days and 30 days. The activity of the amylolytic enzymes in the Kojis mentioned above are shown in Table 8. The total amylolytic activity increased from day 0 to day 10 and remained almost the same over the 30 days. There was no significant difference from day 0 to day 30 with respect to the α-amylase activity. AMG activity increased from day 0 to day 10 but remained almost the same from day 10 to day 30.

TABLE 7. Moisture Content in Koji and the Respective Total Amylolytic Activity

Moisture Content (mL)	Total Amylolytic Activity (Units*)
10	1.8
15	1.7
20	1.3
25	0.8
30	1.0

*Units/mL

TABLE 8. Activity of Amylolytic Enzymes in Koji Over 30 Days

Time (Days)	Total Amylolytic Activity (Units*)	α-amylase Activity (Units*)	AMG Activity (Units*)
0 (fresh)	3.30	2.83	0.47
10 (dry)	3.88	3.02	0.86
20 (dry)	3.76	2.94	0.82
30 (dry)	3.70	2.89	0.81

***Units/mL**

4.1.7 Determination of the Stability of Amylolytic Enzymes in Koji at 30^0C Over 72 Hours

One plate of fresh Koji was made using 10^8 of each spore type in 10 mL Triton X-100 using 15 g corn (3.4). This was ground in a mortar, put into a 250 mL beaker and the volume made up to 200 mL with tap water (pH 4.0 -4.2). After stirring for about 20 minutes on a magnetic stirrer, 10 mL was filtered out and the amylolytic activity assayed.

The rest was covered with aluminium foil and continuously stirred on a magnetic stirrer. This was kept in a 30^0C incubator for 72 hours. Ten mL samples were taken after every 24 hours, filtered and assayed for amylolytic activity over the 72 hours. Shown in Table 9 are the activities of the amylolytic enzymes in the above investigation. The total amylolytic activity increased from 0 hour to 24 hours but decreased at 48 hours. It however increased again at 72 hours. The ∝-amylase activity increased from 0 hour to 24 hours and remained almost the same until 72 hours. The AMG activity increased from 0 hour to 24 hours but decreased at 48 hours. It again increased at 72 hours.

4.1.8 Determination of the Stability of Amylolytic Enzymes in Koji Over 180 Days

Two plates of Koji, using 10^8 of each spore type

TABLE 9. Activity of Amylolytic Enzymes in Incubation at 30^0C Over 72 Hours

Time (Hours)	Total Amylolytic Activity (Units*)	α-amylase Activity (Units*)	AMG Activity (Units*)
0	3.78	2.73	1.05
24	5.60	3.81	1.79
48	5.08	3.80	1.28
72	5.48	3.96	1.52

*Units/mL

in 10 mL Triton X-100 and 15 g corn, were mixed, ground and divided by weight into eight aliquots. Seven of these aliquots were put into small aluminium dishes and heated and dried at 41^{0}C in vacuuo, for 18 hours. They were then cooled to room temperature. Six of these were put into 100 mL beakers, covered tightly with aluminium foil and stored in the dark at room temperature.

Enzymes were extracted from the fresh, and the dry aliquot not stored, in 50 mL total volume of tap water (pH 4.0 - 4.2) and the amylolytic activity of each assayed. One of the dried stored aliquots was taken after every 30 days, enzymes extracted as before and the amylolytic activity assayed over a period of 180 days. Table 10 shows the activity of amylolytic enzymes over 180 days.

The heated and dried Koji of day 0, had higher amylolytic activities than the fresh Koji. From day 0 the dried Koji amylolytic activity decreased gradually until day 180. The activity of the α-amylase was always higher than that of AMG. The AMG was more stable than the α-amylase because whilst at day 180 the α-amylase had decreased by 45%, the AMG had decreased by only about 16%. Total amylolytic activity decreased 32% by day 180.

TABLE 10. The Stability of Amylolytic Enzymes Over 180 Days

Time (Days)	Total Amylolytic Activity (Units*)	α-amylase Activity (Units*)	AMG Activity (Units*)
0 (fresh)	1.86	1.14	0.72
0 (dry)	1.96	1.03	0.93
30	1.91	1.03	0.88
60	1.80	1.00	0.80
90	1.65	0.87	0.78
120	1.64	0.84	0.80
150	1.24	0.84	0.40
180	1.26	0.65	0.61

*Units/mL

4.2 Yeast Inoculum

4.2.1. Effect of Yeast Inoculum Size on the Production of Ethanol In SSF

Six plates of Koji were prepared as before (3.4). Forty five g of corn were weighed into each of six 250 mL beakers. One plate of fresh Koji was added to each beaker and about 150 mL of tap water was added, stirred, and the pH adjusted to 4.0 - 4.2.

Volumes of yeast (strain Y-174), which had been grown for 48 hours were centrifuged at 12,000 rpm, the supernatants decanted. The yeast was resuspended in the same volume of tap water. Percentages by volume of yeast suspension were dispensed in duplicate beakers as follows:

1% = 2 mL

2.5% = 5 mL

5.0% = 10 mL

The volumes in all the beakers above were brought up to 200 mL with tap water, a stirring bar added, loosely covered with aluminium foil and incubated for 72 hours at 30^0C with continuous stirring on magnetic stirrers.

The effect that the yeast inocula size had on SSF is illustrated in Table 11. These are the averages of the duplicate fermentations. It can be seen from Table 11 that

TABLE 11. Yeast Inocula Sizes and the Amount of Ethanol Produced in SSF

Yeast Inoculum Size (%)	% Ethanol (v/v)
1.0	9.65
2.5	9.50
5.0	9.65

the inoculum size of yeast has no significant difference on the amount of ethanol that is produced in SSF.

4.3 Simultaneous Saccharification and Fermentation of Ethanol

The ingredients for the simultaneous saccharification and fermentation (SSF), unless otherwise stated, were 45 g corn, one plate of fresh Koji (3.4), 85% phosphoric acid (to adjust the pH to 4.0-4.2) and 5 mL (2.5%) of a 48 hour washed yeast culture in a total volume of 200 mL.

These were all added at once in a 250 mL beaker and mixed well, then covered by aluminium foil. Mixing provided by a magnetic stirring bar was continued throughout the incubation period of 72 hours at 30^{0}C.

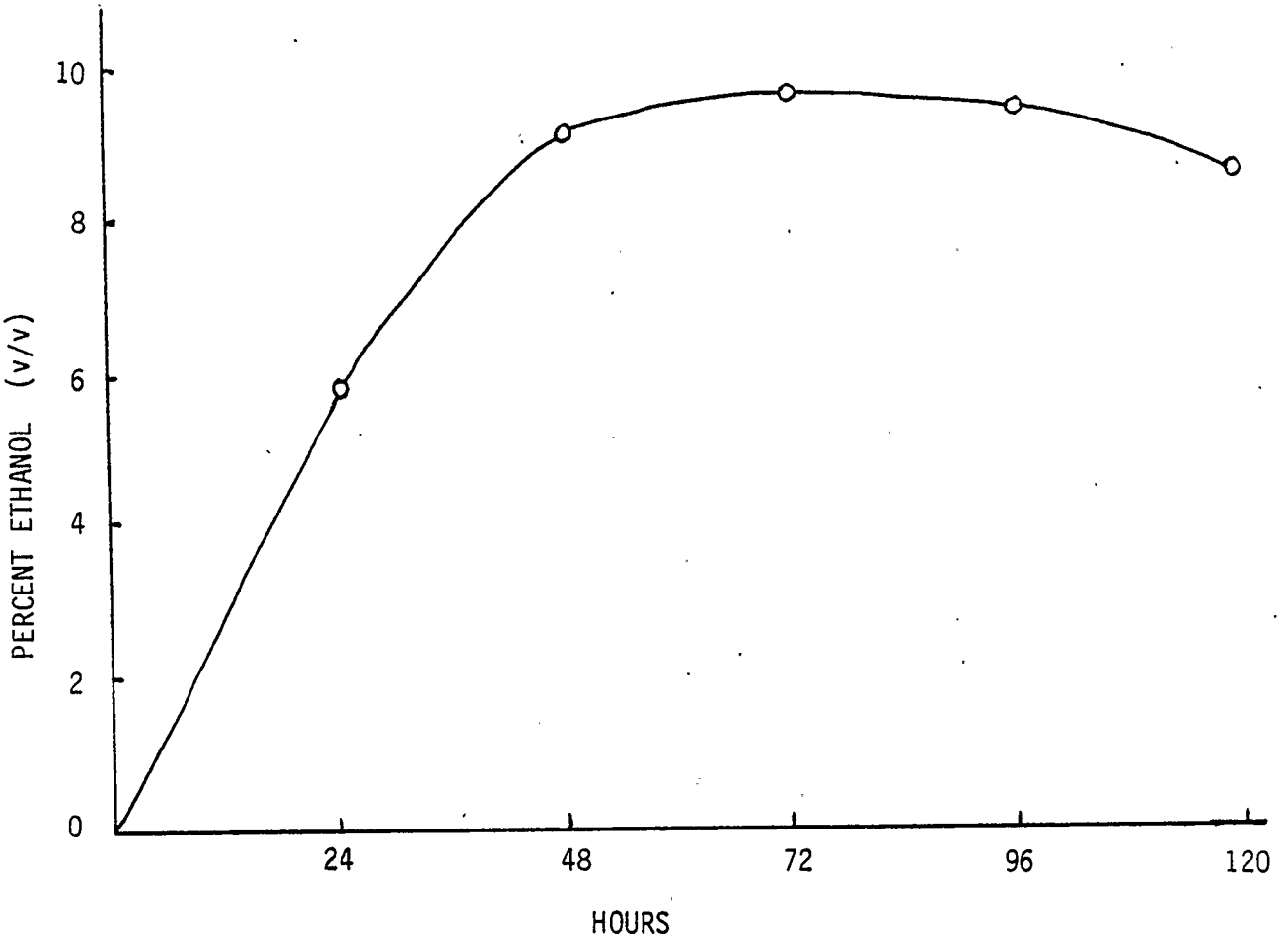
4.3.1 The Course of Ethanol Production in SSF

Two plates of Koji (3.4.), were made and used in two SSF as duplicates (4.3). These were incubated and about 10 mL samples taken from each beaker every 24 hours.

The course of ethanol production in SSF is shown in Figure 2. These are the means of the duplicate fermentations. Ethanol is produced by 24 hours. It is also evident that the rate of production of ethanol until 24 hours is the highest. From then the rate decreases until 72

FIGURE 2 Production of Ethanol in SSF with Time

0 = Production of Ethanol


PERCENT ETHANOL (v/v)
0
2
4
6
8
10
24
48
72
96
120
HOURS

hours. From 72 hours to 120 hours the rate is negative.

4.3.2 Effect of the Amount of Corn on the Production of Ethanol in SSF

Ten plates of Koji (3.4), were made and used in five duplicate SSF experiments (4.3) involving the following weights of corn: 45 g, 50 g, 55 g, 60 g and 65 g.

Table 12 shows the averages of the effect of varying the amount of corn on the production of ethanol in SSF. The theoretical yield of ethanol increased from 45 g of corn to 50 g and started decreasing slowly until 65 g.

4.3.3 Effect of the Amount of Koji on the Production of Ethanol in SSF

Five plates of Koji (3.4), were made. These were ground together in a mortar and duplicates of 5 g, 10 g, and 15 g were weighed into separate 250 mL beakers. Forty five g corn and 150 mL of tap water were added to each and the pH's adjusted to 4.0 to 4.2. Five mL of 48 hour washed yeast culture was added to each of the beakers and the volumes brought up to 200 mL. These were incubated for 72 hours at 30^0C with constant stirring on magnetic stirrers. The samples were filtered, centrifuged at 12,000 rpm and the ethanol contents anyalysed as before. The effect of the amount of Koji on the production of ethanol in SSF is shown

TABLE 12. Amount of Corn and the Ethanol Produced in SSF

Weight of Corn (g)	% Ethanol (v/v)	Efficiency *
45	8.60	87
50	10.15	92.4
55	10.25	84.8
60	10.70	81.2
65	10.70	79.9

***Percent of Theoretical Yield**

in Table 13. These are means of duplicate fermentations. Production of ethanol increased from 5 g Koji through 10 g to 15 g although the increase from 10 g to 15 g was not as much as from 5 g to 10 g. The 15 g sample corresponds to a more than 98% theoretical yield of ethanol.

4.3.4 Effect of Particle Size of Corn on the Production of Ethanol in SSF

Ground corn was used as the control. This was further ground in separate batches in a mortar to pass through different sieve mesh sizes. The corn was ground so the total batch passed through the respective sieve mesh.

The mesh sizes of sieve used were:

I. No. 10 (<2.0 mm)

II. No. 18 (<1.0 mm)

III. No. 35 (<0.5 mm)

IV. No. 60 (<0.25 mm)

Simultaneous saccharification and fermentation trials (4.3), were carried out with each of these corn sizes in duplicate.

The results representing the averages of the duplicate trials are shown in Table 14. It can be seen that there were no significant differences in the ethanol produced in all the corn sizes.

TABLE 13. Amount of Koji and the Ethanol Produced in SSF

Weight of Koji (g)	Percent Ethanol (v/v)
5	7.7
10	9.0
15	9.7

TABLE 14. Particle Size of Corn and the Production of Ethanol in SSF

Sieve Mesh Size of Corn	Percent Ethanol Produced (v/v)
Control	9.62
No. 10 (<2.0 mm)	9.60
No. 18 (<1.0 mm)	9.70
No. 35 (<0.5 mm)	10.00
No. 60 (<0.25 mm)	9.35

4.3.5 Production of Ethanol from Koji in SSF

A duplicate set of Koji (3.4.) was prepared and assayed for the ability to contribute to the production of ethanol by eliminating the addition of corn (4.3.2) prior to fermentation. Koji only in SSF did not produce any ethanol.

4.4 Comparison of Fermentation Technologies

The "Conventional Fermentation" protocol was compared with unconventional methods to evaluate the ethanol producing capabilities of the unconventional methods.

4.4.1 Conventional Fermentation

The "Conventional Fermentation" using heat to gelatinize the corn followed the procedure as in 3.10. The average of the ethanol produced was 8.3% v/v which was 83.9% of theoretical yield.

4.4.2 Simultaneous Saccharification and Fermentation (SSF)

The ingredients and procedure for this trial were as reported in 4.3. Duplicates averaged 8.7% v/v ethanol which was 88% of theoretical yield.

4.4.3 Saccharification Before Fermentation (SBF)

The procedure for this trial was as reported in 3.11. The average alcohol produced was 7.7% v/v which was 77.9% of theoretical yield.

4.4.4 Comparison of Efficiencies

The result, averages of duplicates, of the above methods in the production of ethanol is illustrated in Table 15. Although both "Conventional" and SSF resulted in more than 80% of theoretical yields, SSF was the most efficient process.

4.5 Scale Up of SSF

Koji was prepared as usual (3.4). Corn, 150 g, was put into two big baking pans, which were each inoculated with 100 mL 0.1% Triton X-100 suspension containing 10^8 spores, each, of A. niger and A. awamori. The spore suspensions were well mixed with the corn and spread in thin layers (< 5 mm). The pans were then covered with aluminium foil to retain moisture and incubated for 72 hours at 30^0C.

Two 4 L beakers were used as duplicate fermentation vessels. Each vessel contained fresh Koji cut into small pieces and 450 g corn. About 1800 mL of tap water was added to each of the vessels and the pH adjusted to 4.0 to 4.2.

TABLE 15. Comparison Among Fermentation Technologies

Type of Fermentation	Percent Ethanol (v/v)
Conventional	8.3
SSF	8.7
SBF	7.7

Washed yeast, 50 mL was added and the volumes brought up to 2 L with tap water. These were then covered loosely with aluminium foil and incubated at 30^0C for 72 hours with constant stirring by magnetic stirrers.

The amount of ethanol produced was 9.48% v/v and 9.5% v/v in the duplicates resulting to an average of 9.49% v/v (96% theoretical yield).

4.6 Standardization of Amylolysis During SSF

Because the production of Koji for SSF was highly variable (Figure 1; Tables 4 - 10) the following method was used to standardize amylolysis during SSF. Ten plates of Koji (3.4) were prepared, vacuum dried and assayed for activity on a dry weight basis. One unit of total amylolytic activity was found to be equivalent to 3 g dry Koji (3 g dry Koji in a 200 mL fermentor results in 1 unit of total amylolytic activity per mL). A duplicate series of SSF fermentors using 50 g corn was prepared where the amount of dry Koji varied from 1.5-4.5 g per fermentor (0.5-1.5 units total amylolytic activity). The results are found in Table 16.

These results indicate that at least 1.5 units of total activity per 50 g corn should be used in an idealized SSF fermentor. To ensure amylolytic enzymes are in slight excess 2 units/50 g corn (1 unit/25 g corn) should be used

TABLE 16. Effect of Total Amylolytic Activity on Fermentor Efficiency

Total Amylolytic Activity (Units/mL)	% Ethanol (v/v)	Efficiency*
0.5	5.05	46%
0.75	6.30	57.3%
1.0	7.45	67.8%
1.25	8.85	80.6%
1.5	9.75	88.7%

*Percent of theoretical ethanol

in an idealized SSF fermentor design.

4.7 Determination of Moisture Content of Corn

The procedure for this investigation was as in 3.6. The average of the moisture contents of the various corn particle sizes are shown in Table 17. It can be seen from the table that the moisture content of the corn used was 12.50% of dry weight since this was the moisture content of the smallest particle size.

5.0 DISCUSSION

To make it more competitive with petroleum based liquid fuels the cost of ethanol production by fermentation of corn (or starchy substrates) must be reduced. One method to reduce the cost of production is to lower energy input through elimination of the cooking procedure presently required for starch gelatinization. The use of a Koji amylase system that requires no prior gelatinization of starch seems promising for not only the elimination of this cooking but also maintaining high ethanol yields. The use of crude amylases, instead of purified commercial amylases (frequently those produced by Aspergillus species) for liquifaction and saccharification of starch, which also represents a significant additional expense, in the

TABLE 17. Size of Corn Particles and Moisture Content

Sieve Mesh Size of Corn	Moisture Content (% Dry Weight)
Control	11.74
No. 10 (<2 mm)	11.82
No. 18 (<1 mm)	11.97
No. 35 (<0.5 mm)	12.30
No. 60 (<0.25 mm)	12.50

production of fuel alcohol from starchy materials might also be cost effective.

Early work with this newly developing Koji amylase system has shown some promise (Matsumoto et al, 1982; Weller et al., 1984). As a result, most of the goal of this research was to optimize the production conditions of both the amylolytic enzymes (Koji) and the simultaneous saccharification and fermentation (SSF) using ground corn.

5.1 Production of Koji Amylases

Koji or crude amylases derived from Koji have often been used as saccharifying agents in both cooking (Matsumoto et al., 1982) and noncooking systems (Ueda et al., 1981) to produce ethanol from starchy materials. Unfortunately, no research has been published on the optimization of amylolytic activity in Koji. This research attempted to standardize Koji production, but as the results indicate (Figure 1; Tables 4-10) the amylolytic activities varied widely from batch to batch. However a number of parameters seemed to affect the production of Koji and amylolytic activity.

5.1.1 Effect of Quantity and Particle Size of Corn on Koji Amylolytic Activity

If less than 10 g of corn is used to produce Koji the resulting amylolytic activity is substantially decreased. Table 4 shows there is no major difference in amylolytic activity when 10 or 15 g of corn is used. When less corn is used the growth and production of amylolytic enzymes might be limited. However, if 15 g of corn is used growth and amylolytic activity appears maximized and the amount of excessive substrate is not sufficient to contribute to alcohol formation. In mock SSF (4.3.4) Koji does not provide any glucose to the fermentative process and is probably all assimilated by the aspergilli and the yeast.

Although corn particle size showed no significant effect on amyloglucosidase activity (AMG) in Koji the production of α-amylase seemed to show a significant increase when smaller particles were used as substrate. The results of Table 6 indicate about a two-fold increase in α-amylase activity when particle size of <0.5 mm (35 mesh) are used as substrate. Amylolytic activity could be maximized if Koji was produced on sieved ground corn separating out particles <0.5 mm from the larger particles. In average ground corn at least 30% (Table 18) is <0.5 mm particle size, however, the corn used in this study contained >40% in this size range. An initial separation of

TABLE 18. Sieve Analysis of Ground Corn Feedstock by American Society of Agricultural Engineers Standard S319 (Weller et al., 1984)

Sieve Mesh	Weight percent retained on designated screen
4	0.7
6	4.8
8	8.1
12	10.6
18	18.7
20	8.8
35	17.4
50	10.6
60	8.8
120	9.8
140	1.4
Bottom Pan	0.3
	100.0

ground corn into these two size ranges is easily accomplished in order to maximize amylolytic activity of Koji.

5.1.2 Effect of Spore Inoculum Size on Koji Amylolysis

Increasing the spore inoculum size beyond 10^8, each, of A. niger and A. awamori in 15 g of corn does not result in any significant increase in the production of amylolytic enzymes. The results of varying the spore inoculum size in Table 5 show that less than 10^8 spores limits the amylolytic activity and greater than 10^8 spores does not seem to substantially increase the amylolytic activity. A ratio of 10^8 spores to 15 g corn appears to be ideal for fungal mycelial growth and enzyme secretion.

5.1.3 Effect of Aeration on the Production of Koji Amylases

The decrease in total amylolytic activity of Koji produced in increasing moisture contents (Table 7) could be explained by the fact that the excessive water fills the void spaces between the corn particles. This displacement of air (oxygen), required for growth of these aerobic aspergilli, decreases growth and enzyme production by oxygen limitation. This limitation effect of oxygen content was

also displayed when the aspergilli inoculated corn was not spread into a thin layer (<5mm). Attempts to carry out Koji production and SSF in the same vessel (65 mm diameter) proved unsuccessful because of poor fungal growth and Koji formation when the aspergilli corn mixture mat was too thick to allow for good aeration.

5.1.4 Effect of Drying on Koji Amylolytic Activity

Heating and drying Koji at 41^{0}C increases the amylolytic activity as indicated by the results in Tables 8 and 10. A temperature of 41^{0}C is not high enough to denature either α-amylase or AMG (Bhella and Altosaar, 1984; Mitsue et al., 1979) but may be high enough to cause membrane damage and allow internalized enzymes to be extracted upon rehydration. Another possibility for the increased activity may be due to denaturation of transglycosylase (Weller, 1983) found in aspergilli Koji preparations. Dry Koji stored at room temperature in the dark remains remarkably stable for 120 days (Tables 8 and 10). Fresh Koji actually increases in activity during mock SSF experiments (Table 9). This increase in activity during mock SSF trials is probably due to secretion of internalized preformed enzyme(s).

5.2 Simultaneous Saccharification and Fermentation (SSF)

In simultaneous saccharification and fermentation (SSF) two reactions occur simultaneously:

$$\text{raw starch} \xrightarrow{\text{Koji}} \text{sugar} \xrightarrow{\text{yeast}} \text{ethanol}$$

Thus, the overall rate of ethanol formation is dependent on the rate-limiting step between starch and ethanol. The maximum fermentation rate would be obtained by using a large enough yeast population that could ferment all the sugar as soon as it is produced (Han and Steinberg, 1987).

The insignificant difference between the amount of ethanol produced with respect to the size of the yeast inoculum indicates that the production of ethanol in SSF, using the same amount of corn and Koji (4.2.1, Table 11), is not limited by the size of the yeast inoculum. In batch or continuous culture (Parsons et al., 1984) this yeast, Y-174, is capable of producing 7.0-7.5% v/v ethanol in 24 hours. Since less ethanol (Figure 2) is produced in SSF in the first day (5.8% v/v) this points to starch hydrolysis being the overall rate-limiting reaction. The excess of yeast ensures the glucose is rapidly depleted by conversion to ethanol thus minimizing the possibility of microbial contamination.

To ensure a continuous release of glucose from starch and that a complete amylolysis is accomplished a proper

ratio of amylolytic enzymes to corn is required. Table 16 illustrates this point, a ratio of <1.5 units/mL of total amylolytic activity/50 g corn does not completely amylolize the starch thus reducing fermentation efficiency. At least 1.5 units/mL of total amylolytic activity/50 g corn is required to ensure reasonable fermentation efficiency. This ratio is far in excess of what is theoretically required and probably is related to the accessibility of the starch to the amylolytic enzymes. Perhaps amylolytic activity far in excess of this ratio might not substantially increase the starch hydrolysis rate and only result in comparable fermentation efficiencies (Table 13).

The insignificant difference in the amount of ethanol produced among the different mesh sizes of corn used in SSF (Table 14) could be explained by the fact that the starch in all the ground corn sizes had equal final susceptibilities to the amylolytic enzymes. Initial rates of hydrolysis might have been faster in the smaller mesh sizes because the larger particles, with smaller surface to volume ratios, initially exposed less starch in the endosperm to hydrolysis. Weller et al. (1984) observed that in some instances the germ of the kernel was still attached to the endosperm and this reduced the surface area accessible to amylase activity. The conversion of starch to sugar might have been too slow to maintain initial fermentation rates comparable with smaller particles but with time the starch

became more accessible to the amylolytic enzymes resulting in the total hydrolysis of starch at the end of fermentation, thus producing the same amount of ethanol.

The fact that the amount of ethanol produced in the 2L SSF showed no significant difference from those trials using the smaller volumes (200 mL) shows that this fermentation technology could be transferred to the pilot plant and industrial scales as long as the parameters used are also scaled up to the respective proportions.

Attempts to carry out both Koji production and SSF in one vessel would require redesigning the fermentor by increasing its width two times since the spore-corn mixture would have to be spread in the bottom of the vessel to a depth of about 5 mm for optimum Koji production. If volume ratios were kept constant the redesigned fermentor would only be 1/4 as high as the standard vessel (65 mm diameter x 85 mm). Such a decrease in height would make the conditions more aerobic and prone to increased microbial contamination making this configuration unfeasible.

5.3 Comparison of Fermentation Technologies

The trials comparing fermentation technologies (4.4; Table 14) indicated that SSF is just as efficient as the conventional process and more efficient and less time-consuming than saccharification before fermentation (SBF)

using Koji amylases. All three methods resulted in good theoretical yields but SSF gave all indications it was the superior technology.

A number of studies carried out on SSF indicate this technology is utilizable on a variety of starchy substrates under both sterile (Abouzied and Reddy, 1987) and non-sterile conditions (Ueda et al., 1981; Matsumoto et al., 1982; Weller et al., 1984; Han and Steinberg, 1987; Mikuni et al., 1987). The studies on non-sterile SSF used a variety of amylolytic enzymes: 1. sterile Rhizopus AMG (Matsumoto et al., 1982)

2. purified Chalara paradoxa AMG (Mikuni et al., 1987)
3. sterile Koji (Ueda et al., 1981)
4. non-sterile Koji (Weller et al., 1984; Han and Steinberg, 1987)

All of these studies and the present study (Table 12) illustrate that non-sterile SSF can produce high yields of ethanol from starchy materials. Of these studies the simplest source of amylolytic enzymes proved to be non-sterile fresh or dry Koji (present study; Weller et al., 1984; Han and Steinberg, 1984). This study (Table 14) and those of Matsumoto et ala. (1982) and Weller et al. (1984) demonstrate that non-sterile SSF results in ethanol production efficiencies comparable to the conventential process.

The results of this study indicate that raw ground corn can be used as a fermentation substrate in order to reduce energy costs inherent in the conventional process. The elimination of cooking or gelatinization in the conventional process lowers the energy input substantially and eliminates the capital cost of the cookers. These operating and capital costs can be reduced for both large -and small-scale facilities thus making SSF more competitive.

In SSF all the ingredients are combined in one vessel so fermentation occurred simultaneously with starch hydrolysis. Therefore as soon as the sugar was released the yeast was able to utilize the sugar for ethanol production. The net effect is that the free glucose concentration in the fermentor is always kept to a minimum. Maintaining a low glucose concentration has two beneficial effects:

1. decreases the possibility of microbial contaminants competing with the large number of yeast cells
2. allows the aspergilli in fresh Koji to continue to synthesize amylolytic enzymes in the absence of catabolite repression (Abouzied and Reddy, 1986; Barton et al., 1969)

In addition, combining all the processes into one vessel reduces the handling and equipment costs. Thus, labour, equipment and management costs can be reduced for both large- and small-scale facilities in comparison to those required for the conventional process.

5.4 Commercial Applicability of Non-sterile SSF

As this study indicates the uses of non-sterile SSF could compete well with the conventional process presently in use (Matsumoto et al., 1982), the one factor that will determine whether SSF is widely adopted by industry is whether the cost and availability of mesophilic enzymes is, less than the sum of the cost of the energy required for cooking and the cost of the thermophilic enzymes presently used in the conventional process. Some plants presently using the conventional process have already adopted a semi-SSF process (J. Morrison, personal communication).

In order to minimize microbial contamination of piping and fermentors, these plants only add ∝-amylase during the cooking process. Because few of the major microbial contaminants found in these plants are capable of metabolizing glucose-polysaccharides the pipes leading from the cookers to the fermentor do not become contaminated. A mesophilic AMG is added to the fermentor, so the only place where free glucose is found is in the fermentor where the high numbers of yeast compete out any potential contaminant. During this study, a non-sterile SSF experiment using commercial thermophilic enzymes (3.10) was carried out resulting in 4.6% v/v ethanol (46% efficiency). This experiment further illustrates that non-sterile SSF can even be carried out under sub-optimal conditions.

Mesophilic amylolytic enzymes are quite stable (Table 10; Bhella and Altosaar, 1984) and if they can be produced at costs competitive with the currently used thermophilic enzymes non-sterile SSF would prove to be cost competitive. A central plant producing non-sterile dry Koji (Tables 10 and 15) could prove to be cost effective in supplying both large-and small-scale non-sterile SSF plants. These types of facilities could prove to be an asset in underdeveloped countries that have a cheap source of starchy fermentable crops.

5.5 Future Prospects

The results of this study and other related studies (Matsumoto et al., 1982; Han and Steinberg, 1987) indicate that the only known technology that can lower the cost of ethanol production from starchy materials is the elimination of starch cooking (gelatinization) used in the conventional process by using non-sterile SSF. Since SSF depends on the commercial availability of stable mesophilic amylolytic enzymes this technological advance will not be utilized until such enzymes become available.

In addition to the commercial availability of mesophilic amylolytic enzymes two other methods of providing in situ amylolysis show great promise. The use of yeast cocultures (Abouzied and Reddy, 1987), indicate that using

an amylolytic yeast in addition to a rapid fermenting yeast SSF can be carried out under sterile conditions. In a more recent report, Kim et al., 1988 showed that yeast cells could be transformed with amylolytic heterologous genes and secrete sufficient enzymes to allow for aerobic growth of this fermenting yeast. Both of these studies, using stably transformed (amylolytic enzymes) or naturally occurring yeast cells in either a mono-or co-culture to carry out non-sterile SSF yielding high alcohol beers from crude starchy materials with high theoretical efficiencies, indicate that the ultimate SSF system is possible.

REFERENCES

Abouzied, M.M., and C.A. Reddy. 1986. Direct fermentation of potato starch to ethanol by cocultures of Aspergillus niger and Saccharomyces cerevisiae. Appl. Environ. Microbiol. 52:1055-1059.

Abouzied, M.M., and C.A. Reddy. 1987. Fermentation of starch to ethanol by a complementary mixture of an amylolytic yeast and Saccharomyces cerevisiae. Biotechnol. Lett. 9: 59-62.

Aiba, S., M. Shoda, and M. Nagatani. 1968. Kinetics of product inhibition in alcohol fermentation. Biotechnol. Bioeng. 10: 845-864.

Balls, A.K., and S. Schwimmer. 1944. Digestion of raw starch. J. Biol. Chem. 156: 203-211.

Barnard, G.W. 1984. Liquid fuel production from biomass in developing countries-an agricultural and economic perspective, p.163-178. In W.D. Lee (ed.), Bioconversion systems. C.R.C., Boca Raton.

Barton, L.L., D.R. Lineback, and C.E. Georgi. 1969. The influence of nitrogen and carbon sources on the production of glucoamylase by aspergilli. J. Gen. Appl. Microbiol. 15: 327-344.

Bhella, R.S., and I. Altosaar. 1984. Purification and some properties of the extracellular α-amylase from Aspergillus awamori. Can. J. Microbiol. 31: 149-153.

Blish, M.J., R.M. Sandstedt, and D.K. Mecham. 1937. Action of wheat amylases on wheat starch. Cereal Chem. 14: 605-628.

Brown, H.T., and J. Heron. 1879. Contribution to the history of starch and its transformation. J. Chem. Soc. 35: 596-654.

Calvin, M. 1984. Renewable fuels for the future. J. Appl. Biochem. 6: 6-18.

Carreira, L.H., and L.G. Ljungdahl. 1983. Production of ethanol from biomass using anaerobic thermophilic bacteria, p. 1-29. In W.D. Lee (ed.), Liquid fuel development. C.R.C., Boca Raton.

Corman, I., and A.F. Langlykke. 1948. Action of mold enzymes in starch saccharification. Cereal Chem. 25: 190-201.

Cysewski, G.R., and C.R. Wilke. 1977. Rapid ethanol fermentations. Biotechnol. Bioeng. 21: 1125-1143.

Dermot, A.O.S. 1979. Biotechnology gains brighten resources outlook. Chem. Eng. News 57: 27-29.

Englard, S., and T.P. Singer. 1950. Physiochemical studies on α-amylase. J. Biol. Chem. 187: 213-219.

Fogarty, W.M. 1981. Enzymes relevant to starch processing in alcohol production, p. 19-30. In T.P. Lyons (ed.), A step to energy independence. Alltech Technical Publications, Lexington.

Freeman, G.G., and R.H. Hopkins. 1936. The mechanism of degradation of starch by amylases. Nature of the early fission products. Biochem J. 30: 442-445.

Giri, K.V., V.N. Nigam, and K. Saroja. 1953. Application of circular paper chromatography to the study of mechanism of amylolysis. Naturwissenchaften 40: 484-485.

Goodwin, T.W., and E.I. Mercer. 1982. Carbohydrate biosynthesis, p. 227-271. Introduction to plant biochemistry. Pergamon Press, Toronto.

Hall, C.W., A.W. Farrall, and A.L. Rippen. 1970. Moisture measurements (Food). Ency. Food Eng. 1: 403-502.

Han, I.Y., and M.P. Steinberg. 1987. Amylolysis of raw corn by Aspergillus niger for simultaneous ethanol fermentation. Biotechnol. Bioeng. 30: 225-232.

Kim, K, C.S. Park, and J.R. Mattoon. 1988. High-efficiency, one-step starch utilization by transformed Saccharomyces cells which secrete both yeast glucoamylase and mouse -amylase. Appl. Environ. Microbiol. 54: 966-971.

Kirk - Othmer. 1984. Alcohol Fuels, p. 1-42. In Kirk-Othmer (ed.), Encyclopedia of chemical technology, Supplement Volume. John Wiley and Sons, Toronto.

Kitahara, K., and M. Kurushima. 1950. Comparison of diastatic enzyme systems of several important molds. J. Fermen. Technol. 27: 1-15.

Kneen, E., O.C. Beckford, and R.M. Standstedt. 1941. The starch degradation properties of barley malts. Cereal Chem. 18: 741-754.

Leach, W.H. 1965. Gelatinization of starch, p. 289-307. In W. Paschall (ed.), Starch Chemistry and technology - Fundamental aspects, Volume 1. Academic Press, New York.

Leach, W.H., and T.J. Schnoch. 1961. Action of various amylases on granular starches. Cereal Chem. 38: 34-46.

Lie, S., A.D. Haukel, and J.J. Gether. 1970. Gas chromatographic determination of ethanol. Brygmesteren (Nov.) p. 281-291.

Lu, P., and S. Ma. 1943. Action of wheat ∝-amylase on lotus-rhizome starch. J. Chin. Chem. Soc. 10: 194-199.

Lyons, T.P. 1981. Gasohol, a viable product whose time has come, p. 1-18. In T.P. Lyons (ed.), A step to energy independence. Alltech Technical Publications, Lexington.

Lyons, T. P. 1983. Ethanol production in developed countries. Process Biochem. 18: 18-25.

Matsumoto, N., O. Fukushi, M. Misanobu, K. Katsush, E. Nakajima, and H. Yoshizumi. 1982. Industrialization of a noncooking system for alcoholic fermentation from grain. Agric. Biol. Chem. 46: 1549-1558.

Meyer, K.H. 1952. The past and present of starch chemistry. Experientia 8: 405-444.

Mikuni, K., M. Monma, and K. Kainuma. 1987. Alcohol fermentation of corn starch digested by Chalara paradoxa amylase without cooking. Biotechnol. Bioeng. 19: 729-732.

Mitsue, T., B.C. Saha, and S. Ueda. 1979. Glucoamylase of Aspergillus oryzae cultured on steamed rice. J. Appl. Biochem. 1: 410-422.

Mullins, J.T. 1985. Enzymatic hydrolysis and fermentation of corn for fuel alcohol. Biotechnol. Bioeng. 27: 321-326.

Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.

Park, Y.K., and B.C. Rivera. 1982. Alcohol production from various enzyme-converted starches with or without cooking. Biotechnol. Bioeng. 24: 495-500.

Parsons, R.V., N.G. McDuffie, and G.A. Din. 1984. pH inhibition of yeast ethanol fermentation in continuous culture. Biotechnol. Lett. 6: 677-680.

Pazur, J.H. 1965. Enzymes in synthesis and hydrolysis of starch, p. 133-171. In W. Paschall (ed.), Starch chemistry and technology-Fundamental aspects, Volume 1. Academic Press, New York.

Pazur, J.H., and T. Ando. 1959. The action of an amyloglucosidase of Aspergillus niger on starch and malto-oligosaccharides. J. Biol. Chem. 234: 1966-1970.

Pazur, J.H., and T. Ando. 1960. The hydrolysis of glucosyl oligosaccharides with α-D-(1-->4) and α-D-(1-->6) bonds by Fungal amyloglucosidase. J. Biol. Chem. 235: 297-302.

Pazur, J.H., D. French, and D.W. Knapp. 1950. Mechanism of salivary amylase action. Proc. Iowa Acad. Sci. 57: 203-209.

Pazur, J.H., and K. Kleppe. 1962. The hydrolysis of α-D-glucose by amyloglucosidase from Aspergillus niger. J. Biol. Chem. 237: 1002-1006.

Phillips, L.L., and M.L. Caldwell. 1951. A study of the action of gluc, amylase, a glucose-producing amylase, formed by the mold, Rhizopus delemar. J. Am. Chem. Soc. 73: 3563-3568.

Pool, E.L., and L.A. Underkofler. 1953. Fungal saccharifying agents. Amylolytic factors of bran culture and submerged culture. J. Agr. Food Chem. 1: 87-90.

Pringsheim, H., and F.Z. Loew. 1932. The specificity of sucrases. Physiol. Chem. 207: 241-247.

Rogers, P.L., J.K. Lee, and D.E. Tribe. 1980. High productivity ethanol fermentations with Zymomonas mobilis. Process Biochem. 15: 7-11.

Rothman, H., R. Stanley, S. Thompson, and Z. Towalski. 1981. Fuel production from biomass, p. 38-42. In H. Rothman, R. Stanley, S. Thompson, and Z. Towalski (ed.), Biotechnology. A review and anotated bibliography. Pergamon Press, Toronto.

Sandstedt, R.M., and R.L. Gates. 1954. A comparison of the raw starch digesting capabilities of the amylase systems from four ∝-amylase sources. Food and Res. 19: 190-199.

Schwimmer, S. 1945. The role of maltase in enzymolysis of raw starch. J. Biol. Chem. 161: 219-233.

Skotnicki, M.L., J.K. Lee, D.E. Tribe, and P.L. Rogers. 1982. Genetic engineering of microorganisms for chemicals. Basic Life Sciences 19: 271-290.

Smiley, K.L., D.E. Hensley, M.J. Smiley, and H.J. Gasdorf. 1971. Kinetic patterns of glucoamylase isozymes isolated from Aspergillus species. Arch. Biochem. Biophys. 144: 694-699.

Tubb, R.S. 1984. Genetics of ethanol producing microorganisms. C.R.C. Crit. Rev. Biotechnol. 1: 241-261.

Ueda, S. 1958. Black-Koji mold amylase systems responsible for digesting raw starch, p. 491-494. Proceedings of the International Symposium on Enzyme Chemistry, Vol. 2. Tokyo and Kyoto.

Ueda, S., and S. Kano. 1975. Multiple forms of glucoamylase of Rhizopus species. Die Starke 27: 123-128.

Ueda, S., and Y. Koba. 1980. Alcoholic fermentation of raw starch without cooking by using black-Koji amylase. J. Fermen. Technol. 58: 237-242.

Ueda, S., R. Ohba, and S. Kano. 1974. Fractionation of glucoamylase system from black-Koji mold and the effect of adding isoamylase and ∝-amylase on amylolysis by the glucoamylase fractions. Die Starke 26: 374-378.

Ueda, S., C. T. Zenin, D.A. Monteiro, and Y.K. Park. 1981. Production of ethanol from raw cassava starch by a nonconventional fermentation method. Biotechnol. Bioeng. 23: 291-299.

Walker, G.J., and P.M. Hope. 1963. Action of some α-amylases on starch granules. Biochem. J. 86: 452-462.

Watanabe, K., and T. Fukimbara. 1965. Studies on saccharogenic amylase produced by Aspergillus awamori. J. Fermen. Technol. 43: 690-696.

Weidenhagen, R., and A.Z. Wolf. 1931. Limit of enzymic decomposition and amylase complement. Z. Ver. Dtsch. Zucker - Ind. 80: 866-876.

Weller, C.L. 1983. M.Sc. thesis. Fuel ethanol from raw corn by aspergilli hydrolysis with concurrent yeast fermentation. Department of Food Science, University of Illinois, Urbana.

Weller, C.L., M.P. Steinberg, and E.D. Rodda. 1984. Fuel ethanol from raw corn by aspergilli hydrolysis with concurrent yeast fermentation. Biotechnol. Bioeng. Symp. 13: 437-447.

Wolfrom, M.L., and A. Thompson. 1955. Degradation of amylopectin to nigerose. J. Am. Chem. Soc. 77: 6403.

Yamasaki, Y., and S. Ueda. 1951. Action of black-Koji amylase on raw starch. J. Agric. Chem. Soc. Jpn. 24: 181-185.