Bioethanol Production from Wheat Milling By-products

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Abstract

The state of the art of starch hydrolysis and fermentation technologies to produce ethanol from wheat by-products was evaluated. Two samples of low-grade wheat flour, namely Low-Grade 1 (LG1) and Low-Grade 2 (LG2), with different carbohydrate and fibrous content, were used as substrates. The samples were liquefied using various concentrations of α- or β-amylase, in order to optimize the production of fermentable sugars; the enzyme α-amylase revealed higher performance. After liquefaction, the simultaneous saccharification and fermentation was conducted in a jar fermentor. Glucoamylase was used for saccharification, and *S. cerevisiae* (dried baker's yeast), for fermentation simultaneously. Sterile samples were withdrawn regularly for analysis. Glucose was consumed promptly in both cases, LG1 and LG2; meanwhile, the ethanol production was considerably higher in LG1 (38.6 g/L), compared to LG2 (24.9 g/L). The substrate containing LG1 revealed higher potential as substrate for ethanol production.

Low-grade wheat flour (LG) was used as substrate for fermentation, at three different levels. Slurries containing 100, 200 or 300 g-flour/L were prepared in distillate water. The samples were initially liquefied using α- amylase (400 U/g-flour) at 55°C for 2 h, stirring at 100 rpm. After adjusting the pH of liquefied mash to 4.5, the simultaneous saccharification and fermentation (SSF) was conducted in a jar fermentor, using glucoamylase (200 U/g-flour) for saccharification, and *Z. mobilis* NBRC 13756 was used as seed culture ($2x10^4$ cells/g-flour) for fermentation; the anaerobic SSF was conducted at pH 4.5, 35°C for 48 h, with mechanical agitation at 100 rpm. Samples were withdrawn periodically for analysis. Cell density, along with other kinetic parameters of microbial growth, the ethanol yield and productivity were

also evaluated. When the intermediate substrate concentration (200 g/L) was utilized, a peak ethanol production (51.5 g/L) was obtained after 24 h fermentation, with an average yield of 0.26 g-ethanol/g-flour, which is about 4.8 times higher than the ethanol yield from sugar cane.

The performance of two wheat milling by-products, low-grade wheat flour (LG) and wheat bran (WB), as substrates for fermentation was evaluated and compared to wheat flour (WF), used as reference. Slurries containing 200 g/L of each were prepared separately and hydrolyzed using 400 U/g-flour of α-amylase (for LG and WF) or cellulase (for WB). After liquefaction, the enzyme glucoamylase was added for saccharification (200 U/g-flour), *Z. mobilis* NBRC 13756 was used as starter culture, and the SSF was conducted in a 2-L jar fermentor. In order to evaluate the fermentation performance of each wheat product, the ethanol production (P, g/L), ethanol productivity (Q, g/L·h), overall volumetric ethanol productivity (Q_v, g/L·h), ethanol yield ($Y_{P/S}$, g-ethanol/g-substrate), and the residue formation (R_f, g-dry solids) were considered. After 28 h of SSF, the maximum ethanol production from LG was 51.4 g/L, which is approximately 2.5 fold that obtained from WB. The ethanol productivity from LG increased continuously during the initial phase of fermentation process, reaching up to 4.4 g/L·h after 6 h, meanwhile in case of WB the peak productivity (1.2 g/L·h; which is lower than LG and WF) was obtained after 12 h of SSF. Using LG as substrate, the overall volumetric ethanol productivity was 2.72 g/L·h, and the ethanol yield was 0.17 g/g-substrate, which is about 8 fold the yield from WB, and relatively higher than other agricultural crops commonly used as feedstock for fuel ethanol production, such as sugar cane (0.06 g/g) or cassava (0.14 g/g).

Kinetics parameters of batch or fed-batch fermentations processes in a small-scale bioreactor were evaluated. Specific fermentation rates, such as specific growth rate (μ), specific ethanol production rate (*qp*) and specific glucose consumption rate (*qs*), as well as yield parameters, such as ethanol yield ($Y_{p/s}$) and biomass yield ($Y_{x/s}$) were determined. On this concern, reference processes were accomplished, using different concentration of glucose in the substrate, in order to evaluate the effects of the substrate feeding mode (batch or fed-batch) on the fermentation. In case of processes conducted in batch mode, the substrate containing 50 g/L of glucose resulted in *c.a.* 18 g /L of ethanol, representing an overall yield of 0.36 g-ethanol/g-glucose which is about 70 % of theoretical yield. As for the substrate containing 100 g/L of glucose, *c.a.* 35 g/L of ethanol were obtained, yielding 0.35 g-ethanol/g-glucose. In case of fed-batch processes, a final ethanol concentration of *c.a.* 48 g/L, which is nearly 1.3 times the ethanol production when 100 g/L of glucose was used as substrate for batch fermentation. Compared to batch processes, considerably higher values of specific glucose consumption rate, as well as specific ethanol productivity, were obtained when fed-batch processes were used for fermentation.

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Nomenclature

LG:	Low-grade wheat flour
WB:	Wheat bran
WF:	Wheat flour
SHF:	Separate hydrolysis and fermentation
SSF:	Simultaneous saccharification and fermentation
M_{cg}:	Mass of glucose consumed (g)
M_{pe}:	Mass of produced ethanol (g)
P:	Ethanol production (g/L)
Q:	Ethanol productivity (g/L·h)
Q_v:	Overall volumetric ethanol productivity (g/L·h)
q_s:	Specific glucose consumption rate (g/g·h)
q_p:	Specific ethanol production rate (g/g·h)
R_f:	Residue formation (g-dry solids)
S:	Glucose concentration (g/L)
X:	Biomass concentration (g-dry weight/L)
Y_L:	Liquefaction Yield (g-maltose/g-flour)
$Y_{P/S}$:	Ethanol yield (g-ethanol/g-flour)
$Y_{p/s}$:	Ethanol yield[a] (g-ethanol/g-glucose)
$Y_{x/s}$:	Biomass yield (g-biomass/g-glucose)
μ:	Microbial growth rate (1/h)
μ_x:	Specific growth rate (1/h)
g:	Generation time (h)

[a] $Y_{p/s}$ is the ethanol yield per weight of glucose, obtained during the reference fermentation experiment (chapter 6); in case of $Y_{P/S}$ the basis for calculation is per weight of flour.

Chapter 1

General Introduction

Ethanol and ethanol-gasoline mixtures have been considered for use as fuel since the early days of the automobile. In the past, the abundant and less expensive petroleum supply prevented the extensive use of ethanol as fuel, but in the last few decades the general public has become aware of and concerned about the increasingly expensive petroleum supply. Interest in extending gasoline supplies with ethanol-gasoline mixtures has increased greatly (Klass, 1981).

Ethanol has been produced by anaerobic yeast fermentation of simple sugars since early recorded history. These fermentations used the natural yeast found on fruits and the sugars of these fruits to produce wines. Current practices utilize bacterial and fungal amylases to efficiently hydrolyze grain or tuber starch to glucose for fermentation to ethanol.

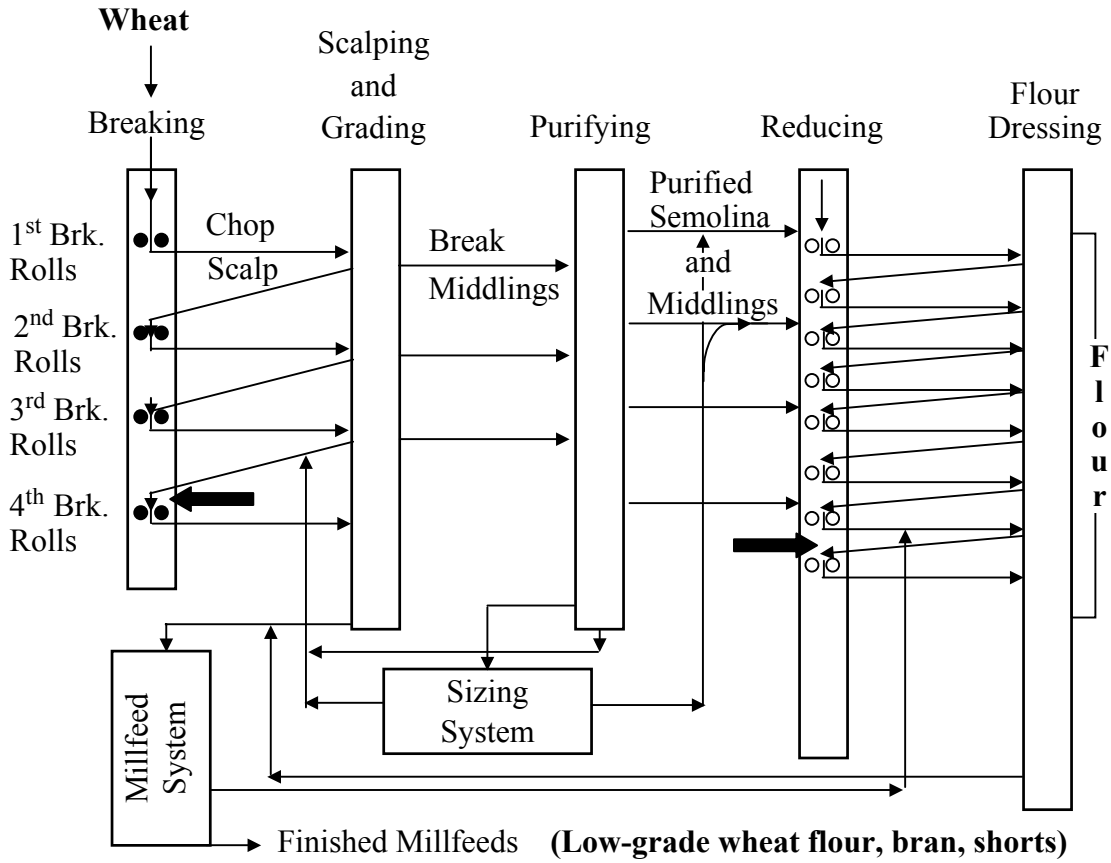
Cereal grain starch is normally a mixture of two types of polymers: amylase, a linear glucose polymer composed of glucose units linked by α-1,4 glycosidic bonds, and amylopectin, a branched polymer. The branch points in amylopectin are α-1,6 bonds.

Industrial processes generally involve the gelatinization of starch-containing raw material with steam, and subsequent liquefaction with α-amylase to dissolve and dextrinize starch carbohydrates; this treatment is referred to as cooking. Then, the resulting crude mash is saccharified with glucoamylase, and fermented with ordinary yeast. Finally, the fermented mash is separated into alcohol and stillage. Ethanol is concentrated using conventional distillation, then is dehydrated. Anhydrous ethanol is blended with denaturant and is ready for shipment into the fuel market (Olsen, 2001).

1.1 Wheat Milling By-products

Wheat has been used as supplementary staple food for thousands of years, since people first began to settle in permanent communities. Wild cereal grassers were the early food grains. As civilization progressed, people learned to select seeds of superior plants.

The developments that led to the present concept of a gradual reduction process in milling occurred during the latter half of the 19th century, with the invention of the middlings purifier, in France in 1860. Subsequently, in the 20th century, the most notable achievements in milling have taken place in materials handling, further refinement, improvements of existing milling machinery and automation of the milling process.



Filled arrows indicate the tail end of breaking rolls and size reduction system, where LG and WB are generated.

Figure 1.1 Flowchart of wheat milling process (adapted from Pomeranz, 1988)

Figure 1.1 shows schematically a simple dry milling process with four break passages, grading, purification and eight reduction passages. Low-grade wheat flour (LG) and wheat bran (WB) are by-products generated at the tail end of breaking rolls and size reduction system (indicated by filled arrows), and separated at the millfeed system.

Those two by-products, along with germ, are the major wheat milling by-products, and they are of considerable economic significance to the miller (Pomeranz, 1988). LG and WB are mainly composed of kernel outer parts of, such as outer pericarp, aleurone layer and starchy endosperm (Figure 1.2) resulting in a varied composition, depending on which stage of milling process it was extracted (Hoseney, 1986).

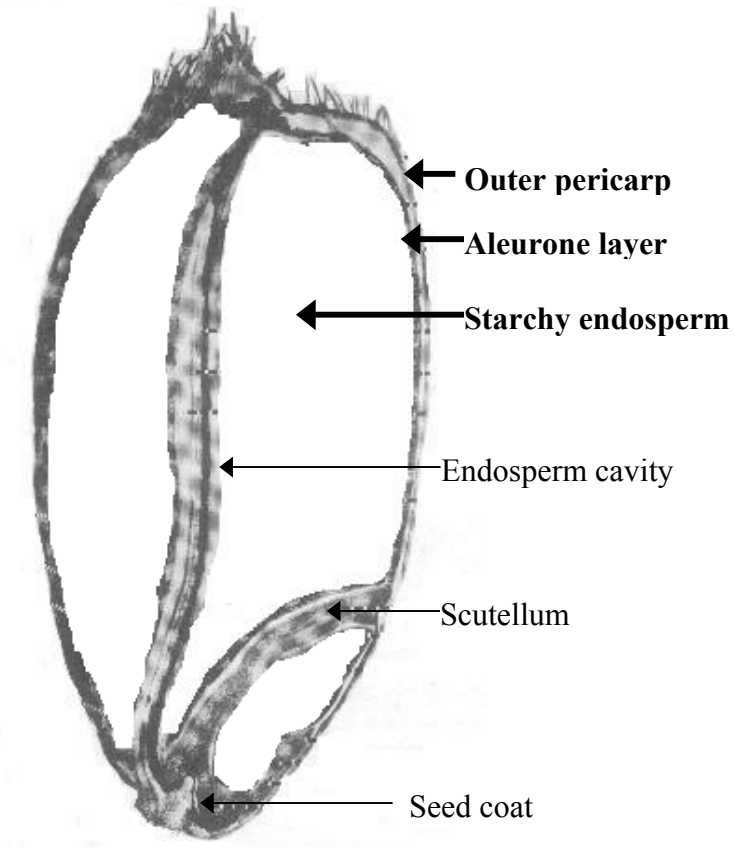
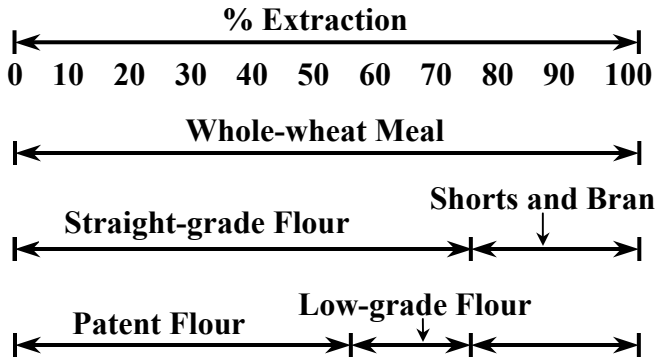


Figure 1.2 Schematic of wheat grain bisected longitudinally through the crease (Adapted from Pomeranz, 1988b)

In Brazil, the total amount of wheat flour produced in the year 2000 was about 6.8 million tons (FIBGE, 2001), from which, about 5 % represent the LG (Hoseney, 1986).

The extraction yield of various products is presented in Fig. 1.3. Though the most LG have been used as feed, a little amount is used as adhesive agents as well. WB is used in the food industry mainly as a source of dietary fiber or metallic ions, such as Ca, Mg and Fe. Most recently, the interest on the phytic acid content in WB has been increasing (Thompson, 1992; Jenab, 1998).



Whole-wheat meal is 100% extraction (line 2) but can be divided into straight-grade flour plus shorts and bran (line 3). The straight-grade flour can be further divided into patent and low-grade flours (line 4).

Figure 1.3 Extraction yield of various wheat milling products

(Adapted from Hoseney, 1986)

Amongst the various wheat milling by-products, WB is the one produced in larger amounts. Recently, it has been utilized for different purposes, *e.g.* as substrate for α-amylase production (Haq *et al*., 2003), or as a source of dietary fiber (Miguel *et al*., 1999). Furthermore, some references about ethanol production from raw wheat flour (Montesinos *el al*., 2000; Sharma *et al*., 2002) and damaged wheat grains (Suresh *et al.*,

1999) are also available. However, very few reports on the usage of wheat milling by-products for ethanol production were found in the literature.

Ethanol from grains is assumed to be produced by the dry milling process, in which starch in grain is converted to dextrose, and then ethanol is produced in fermentation and separated in distillation (Kim *et al*., 2004).

1.2 Amylolytic Enzymes

The ethanol-fermenting microorganisms, such as *S. cerevisiae* and *Z. mobilis* are lack of amylolytic enzymes and unable to directly convert the starch into ethanol (Ang *et al*., 2001). Considering that the main potential feedstock for producing bioethanol are composed of carbohydrates, which include starch, cellulose and hemicelluloses, among others, the use of enzymes to break down these oligosaccharides into easily fermentable sugars is a requirement, previous to conducting the fermentation (Kim *et al*., 2004).

Microorganisms produce two types of amylase: endo-amylases (α-amylases), which attack α-1,4 glycosidic bonds of starch polymers at random points along the polymer chain, and exo-amylases (*e.g.* glucoamylase or β-amylase), which hydrolyze units of glucose or maltose from the nonreducing end of the starch polymer.

α-Amylases are widely distributed throughout nature, produced by bacteria, fungi and germinating cereal grains, but only bacterial enzymes exhibit the high temperature stability needed for commercial starch hydrolysis (Fiechter, 1992). Due to high viscosities and mass transfer problems, industrial liquefaction is carried out at the highest possible temperature.

Table 1.1 shows some characteristics of the enzymes most commonly used for starch hydrolysis.

Table 1.1 Characteristics of various enzymes used for starch hydrolysis

Enzyme	Type	Source	Amount	Activity
α-amylase	Liquefying	*B. subtilis*	0.06 % (w/w) of starch	Decreases viscosity (Cleaves α-1,4, pH 5.5, 70 °C)
		B. licheniformis	0.06 % of starch	Decreases viscosity (92 °C)
		Barley malt	0.5-1.0 % of grain	Decreases viscosity (60 °C)
β-amylase	Saccharifying	Barley malt	2.0 % of grain	Generates maltose (Cleaves α-1,6, pH 5.5, 60 °C)
Glucoamylase	Saccharifying	*A. niger*	0.18 % of starch (1.7 L/ton)	Generates glucose (Cleaves α-1,6, pH 5.0, 60 °C)

Source: Fiechter (1992); Olsen (2001)

Typically, using the α-amylase from *B. licheniformis*, the starch slurry along with calcium ions are incubated at 100 °C for 5 to 10 min, then kept at 95 °C for 1 to 2 h (Keneally *et* al., 1986). The liquefaction is essential for several industries; glucopolymers with varying degrees of polymerization are utilized in papermaking, textile preparation, brewing and fermentation. Liquefaction is a preliminary step for saccharification, by which large quantities of D-glucose can be produced from inexpensive sources.

1.3 Fermentation Processes

Ethanol has been produced by anaerobic yeast fermentation of simple sugars since early recorded history. These fermentations used the natural yeast found on fruits and the sugars of these fruits to produce wines. Beer fermentations made use of the amylases of germinating grain to hydrolyze the grain starches to ferment sugars. Current practices utilize bacterial and fungal amylases to efficiently hydrolyze grain or tuber starch to glucose for fermentation to ethanol (Klass, *et al*, 1981).

Ethanol can be produced by biologically catalyzed reactions. In much the same way that sugars are fermented into beverage ethanol by various organisms including yeast and bacteria, sugars can be extracted from sugar crops, such as sugar-cane, and fermented into ethanol. For starch crops such as corn, starch is first broken down to simple glucose sugars by acids or enzymes, known as amylases. Acids or cellulase enzymes similarly catalyze the breakdown of cellulose into glucose, which can be then fermented to ethanol. The hemicellulose fraction of biomass is broken down into various sugars, e.g. xylose, in the presence of acids or enzymes known as xylanases; conventional organisms cannot ferment many of the sugars derived from hemicellulose into ethanol with reasonable yields. However, recently new technologies capable of efficiently convert hemicelluloses into ethanol are under development.

Basically, two different processes can be used to produce ethanol from starch crops: dry milling and wet milling. In dry milling, the feed material is ground mechanically and cooked in water to gelatinize the starch. Enzymes are then added to break down the starch to form glucose, which yeasts ferments to ethanol. In wet milling,

the insoluble protein, oil, fiber, and some solids are removed initially, remaining only a slurry of starch fed to the ethanol production step.

Biological processing offers a number of advantages for converting biomass into biofuels. First, the enzymes used in bioprocessing are typically capable of catalyzing only one reaction, and so formation of unwanted degradation products and by-products is avoided. Additionally, biological transformations occur at near-ambient pressures and temperatures, so that the overall production cost is modest. Furthermore, material not targeted for conversion can pass through the process unchanged and be used for other applications. Finally, biotechnology and bioprocessing are new evolving areas with a demonstrated ability to dramatically alter a process and improve economics. Thus, former hurdles to developing cost-effective technologies for producing ethanol from starch crops and cellulosic biomass may well be overcome (Johansson, *et al*., 1993).

1.3.1 Separate Hydrolysis and Fermentation

The Separate Hydrolysis and Fermentation (SHF) process uses distinct process steps for starch hydrolysis and glucose fermentation (as described in Figure 4.1). The primary advantage of this configuration is that starch hydrolysis and sugar fermentation can be treated separately, thus minimizing the interactions between these steps. However, α-amylases are often inhibited by the accumulation of sugars, and considerable efforts is still needed to overcome this end-product inhibition, which impedes to achieve reasonable ethanol concentrations at high rates and with high yields even at high enzyme loadings (Borzani, *et al*., 1998).

In SHF the starch containing material is initially hydrolyzed by the action of amylolytic enzymes: α-amylase (for liquefaction) and glucoamylase (for saccharification). After complete hydrolysis, the fermentation is conducted as single step, in separate.

1.3.2 Simultaneous Saccharification and Fermentation

The sequence of steps for the Simultaneous Saccharification and Fermentation process (SSF) is virtually the same as for SHF, except that saccharification and fermentation are combined in one vessel (as described in Figure 4.4). The presence of yeast or bacteria along with enzymes minimizes the sugar accumulation in the vessel, and because the sugar produced during starch breakdown slows down α-amylase action, higher rates, yields and concentrations of ethanol are possible using SSF rather than SHF, at lower enzyme loading. Additionally, the presence of ethanol makes the mixture less vulnerable to contamination by unwanted microorganisms, which is a frequent burden in case of industrial processes (Montesinos, *et al*., 2000; Roble, 2003).

In this process, the saccharification of sugars released during starch hydrolysis (mainly maltose) is conducted simultaneously with fermentation. Immediately after liquefaction by α-amylase, the enzyme glucoamylase is added to the slurry, concomitantly with yeasts, and the SSF is conducted in a single reactor.

1.4 State of the Art on Bioethanol Production

The principal candidates for transportation fuels from renewable sources are ethanol, methanol, hydrogen manufactured from biomass, and hydrogen produced by water electrolysis using renewable sources of electricity. Measured in energy terms (dollar per giga Joule) all these fuels will probably cost more to produce than petroleum-based transport fuels in the early years of the 21st century. When an alternative fuel is considered as part of a system designed and optimized for that fuel, the economic performance will often be much better than for the case where the alternative fuel is simply substituted for gasoline in an internal combustion engine vehicle optimized to run on gasoline (Johansson, 1993).

With the search for alternative renewable energy sources, biofuels are fast becoming a viable solution, as they are non-fossil fuels from a renewable agricultural source, resulting in cleaner combustion. Liquid biofuels are mainly developed as a vehicle fuel. At present, the major players are bioethanol and biodiesel (methyl ester of vegetable oils, typically rapeseed or sunflower oil). Because of the increasing demand for fuel ethanol, there is a need to search for high yielding processes and easily accessible technology for the production of ethanol at reduced cost (Sree, *et al*., 2000).

Of all biofuels, ethanol has been trusted as an alternate fuel for the future and is already produced on a fair scale (about 14-26 million tons worldwide, and is easily applicable in present day internal combustion engine vehicles, as mixing with gasoline is possible. About 90% of all ethanol consumed is derived from sugar or starch crops by fermentation; the rest is produced synthetically. The bulk of the production is located in Brazil and the USA, which account for 62 % of world production (Kim, *et al*., 2004).

Brazil is the pioneer in large-scale motor fuel ethanol production through the fermentation of sugar cane molasses by yeast (Rosillo, *et al*., 1998), producing in the year 2004 about 14.2 billion L of bioethanol (Licht, 2005), most of which is fermented using hexose sugars present in cane syrup (Monte Alegre, *et al*, 2003). The substitution of sugar cane based bioethanol for gasoline in passenger cars and light vehicles in the country is one of the largest commercial biomass-to-energy programs in existence today (Goldemberg, *et al*., 2004).

Basically, in the United States ethanol is produced by fermenting and distilling starch crops that have been converted into simple sugars, and the major feedstock for this fuel include corn, barley and wheat. In the year 2003 the fuel ethanol production in the country was approximately 10.6 billion L (Norimatsu, 2005).

Besides those two major players in the world bioethanol production scenario, various countries have been increasing their production, such as India (where bioethanol is basically produced from sugar cane), Thai (using sugar cane or cassava), France (sugar beet or wheat), China (corn), Canada (wheat), among others. The amount of sugar cane produced in India during the harvest 2002/2003 was comparable to that produced in Brazil, about 300 million tons (Daishou, 2004).

In Japan, since August, 2003 the Ministry of Industry and Economy regulated the use of a blend composed of 3 % ethanol and 97 % gasoline (v/v) (E3) as motor fuel for passenger cars, and in October of that year the Ministry of the Environment launched the "Road Map" referring to this blend. As for now, most of research projects undergoing in Japan utilize lignocellulosic biomass to produce bioethanol (Daishou, 2004; MOE, 2003).

1.5 General Objectives

Nowadays there are many studies available in the literature concerning about ethanol conversion systems from wheat products. Unfortunately, most of them are based on the utilization of raw wheat flour (Montesinos, *et al*., 2000; Favela-Torres, *et al*., 1988) or damaged wheat grains (Suresh, *et al*., 1999). Base on that, the originality of this work relies on the utilization of industrial by-products for bioethanol production, more specifically from wheat milling industry. In order to develop a fermentation process suitable for ethanol production from wheat milling by-products, the overall objectives of the present work were as follows:

(1) To evaluate the suitability of wheat milling by-products as substrate for bioethanol production.

(2) To determine the most suitable enzyme, activity, temperature and hydrolysis time, in order to optimize the starch liquefaction, increasing the amount of fermentable sugars released.

(3) To develop a fermentation process capable of efficiently produce high levels of ethanol from wheat milling by-products, considering the most suitable microorganism, and the association of saccharification and fermentation processes.

(4) To present a state of the art on worldwide fuel ethanol production and future trends.

Chapter 2

Starch Liquefaction Optimization

2.1 Introduction

The hydrolysis of starch by amylases at relatively high temperatures is a process known industrially as liquefaction (Fiechter, 1992). The factors that affect the enzymatic hydrolysis of starch include substrates, enzyme activity, and reaction conditions (temperature, pH, as well as other parameters) (Sun *et al*., 2002).

In this study two different commercial enzymes were utilized for starch hydrolysis: α-amylase and β-amylase, and their efficiencies were compared. α-amylase hydrolyzes starch molecules randomly at α-1,4 glycosidic linkages to yield maltose molecules (disaccharides of α-glucose). Meanwhile, β-amylase is a exo-amylase, which hydrolyzes units of glucose or maltose from the nonreducing end of the starch polymer.

The objective of this study was to optimize the hydrolysis of starch contained in LG, in order to increase the liquefaction yield, releasing more maltose for posterior saccharification, and subsequently improving the overall ethanol production.

The effect of several experimental conditions on the hydrolysis performance was evaluated, considering the following variables:

- Enzyme activity: 100 U/g-flour or 200 U/g-flour were utilized.
- Temperature: the hydrolyses were conducted either at 55°C or 75°C.
- Time: the hydrolysis process was conducted up to 2 h.
- Type of enzyme: α-amylase or β-amylase.

2.2 Material and Methods

2.2.1 Raw Material

Two different lots of sample, namely low-grade wheat flour 1 (LG1) and low-grade wheat flour 2 (LG2), were provided by Nisshin Flour Milling Co., Japan. In LG1 the starch content was higher than in LG2, but the latter was rich in fibrous material (Table 2.1).

Table 2.1 Average chemical composition (%) of low-grade wheat flour

Flour	Component (%)					
	Moisture [a]	Starch	Ash	Fiber [b]	Protein [b]	Others
LG 1	14.0 ± 0.15	15.6 ± 1.30	2.7 ± 0.01	0.8	15.0	51.9
LG 2	14.0 ± 0.38	10.4 ± 2.27	3.2 ± 0.05	5.0	16.5	50.9

[a] Mean value ± Standard Deviation (n = 3)
[b] Data provided by Nisshin Flour Milling Corp.

2.2.2 Microorganisms and Culture Media

Dry baker's yeast, *Saccharomyces cerevisiae*, commonly used in bakery and brewery industries (Saf-Instant Lesaffre, Marcq-France) was used as ethanol producing yeast strain.

2.2.3 Enzymes

Two different commercial enzymes were utilized for starch liquefaction. α-Amylase (EC 3.2.1.1, 51 U/mg, Sigma, USA) from *Bacillus* species, and β-amylase (EC 3.2.1.2, 19 U/mg, Sigma, USA) from barley, were used in separate, and their performance was compared, aiming to optimize the final maltose concentration.

2.2.4 Starch Liquefaction Optimization

An optimization of temperature and enzyme activity was performed. It consisted of four experiments (for each sample: LG1 and LG2, in separate): the hydrolysis was conducted at 55°C or 75°C, with two levels of enzyme activity: 100 U/g-flour or 200 U/g-flour. The performance during starch hydrolysis was evaluated based on the maltose production and the liquefaction yield (Y_L) (g-maltose/g-substrate) for each substrate, enzyme and experimental conditions; the liquefaction yield was calculated according to equation 2.1, where M indicates the maltose concentration.

$$Y_L = \frac{(M_{final} - M_{initial})\ (g)}{Substrate\ (g)} \qquad (2.1)$$

Initially 1 Lslurries containing 100 g-flour/L were prepared in distilled water, the enzymes were added (in separate, as described above) and the samples were hydrolyzed at the suitable temperature for 2 h with mild agitation (100 rpm).

2.2.5 Fermentability Test

After the liquefaction of LG1 and LG2, in separate, under the optimum conditions determined above, the pH of hydrolyzed slurries was adjusted to 4.5, glucoamylase (200 U/g-flour) (Chi *et al*., 1993) and dry baker's yeast (10 g/L) were added concomitantly for SSF. The mixture was aseptically transferred to a 2 L fermentor (MDL 200 B.E. Marubishi, Japan) (see details in Figure 3.1), which was previously sterilized. The fermentor is equipped with temperature and pH automatic controls. NaOH 3M was used to maintain the pH 4.5. The process was conducted at 35°C for 48 h, with mild agitation (100 rpm). Nitrogen gas was bubbled at 100 mL/min into the reactor, to assure the anaerobic environment.

The SSF performance was evaluated based on the ethanol yield ($Y_{P/S}$) (g-ethanol/g-substrate), obtained using Equation 2.2.

$$Y_{P/S} = \frac{Ethanol\ produced\ (g)}{Substrate\ (g)} \qquad (2.2)$$

2.2.6 Kinetics of Yeast Growth

When dealing with microbiological processes, it is a key point to know the growth kinetics of the microorganisms. Thus, this experiment was conducted in order to determine the generation time (also called doubling time) for *S. cerevisiae*.

2.2.6.1 Pre-cultivation of Dry Baker's Yeast

Dry baker's yeast (10 g/L) was inoculated in YM broth (pH 6.0), which contained (in g/L): glucose, 10; peptone, 5; yeast extract, 3 and malt extract, 3, in distilled water. Cultures were incubated at 28°C for 24 h.

2.2.6.2 Simultaneous Saccharification and Fermentation

The yeast starter culture (10 mL) was inoculated into 1 L of slurry containing 100 g/L of LG1 and the SSF was conducted as described at the fermentability test (2.2.5). Aliquots were withdrawn at determined intervals of time, plated into YM agar, incubated at 28°C for 48h, and the number of colonies (N, CFU/ml) was assessed.

2.2.6.3 Evaluation of Kinetic Parameters

The microbial growth rate (μ) was calculated by linear regression of the logarithmic number of yeast cell (Log N) during the exponential growth phase (Moon, 2005), and equation 2.3.

$$Slope = \frac{\mu}{2.303} \qquad (2.3)$$

The generation time (g) was calculated according to equation 2.4 (Barker, 1998).

$$\mu = \frac{ln\ 2}{g} \quad (2.4)$$

2.2.7 Analytical Methods

Samples were withdrawn regularly for analysis; all samples were centrifuged at 4,000 rpm for 20 min. Glucose, maltose and ethanol concentrations were analyzed using HPLC, as previously described by Shiiba, *et. al.* (1993). After centrifugation, the supernatant was filtered through chromato-disk filters (pore size = 0.45 μm). The HPLC system used was JASCO consisting of a pump PU-980, detector RI-930, sampler AS-950 (20 μL injection loop), equipped with a column Sugar KS-801 (Shodex Co., Japan) stabilized at 80°C; the eluent used was ultra pure water, at a flow rate of 0.6 mL/min and elution time 30 min. Reducing sugar content was analyzed using the 3,5-Dinitrosalisylic acid (DNS) method (Bernfeld, 1955), and the initial starch content in LG was analyzed using the phenol-H_2SO_4 method (Dubois, 1956). Total nitrogen was analyzed using the Kjeldahl method, modified by Udy (1956); crude protein quantity was expressed using the conversion factor 5.7. Crude fiber was determined as neutral detergent fiber (Van Soest, 1963).

2.3 Results and Discussion

Taking into account that yeasts are not able to ferment polysaccharides, the liquefaction is a preliminary step essential for ethanol production, consisting of internal hydrolysis of α-1,4-glucans (mainly starch, in case of LG) by amylolytic enzymes, lowering the slurry viscosity, releasing disaccharides such as maltose, for posterior saccharification and fermentation (Aziz, 2002).

2.3.1 Optimization of Starch Liquefaction

This experiment was conducted up to 2 h, using two different levels of enzyme load 100 or 200 U-α-amylase/g-flour, at 55 or 75°C, as shown in Figure 2.1.

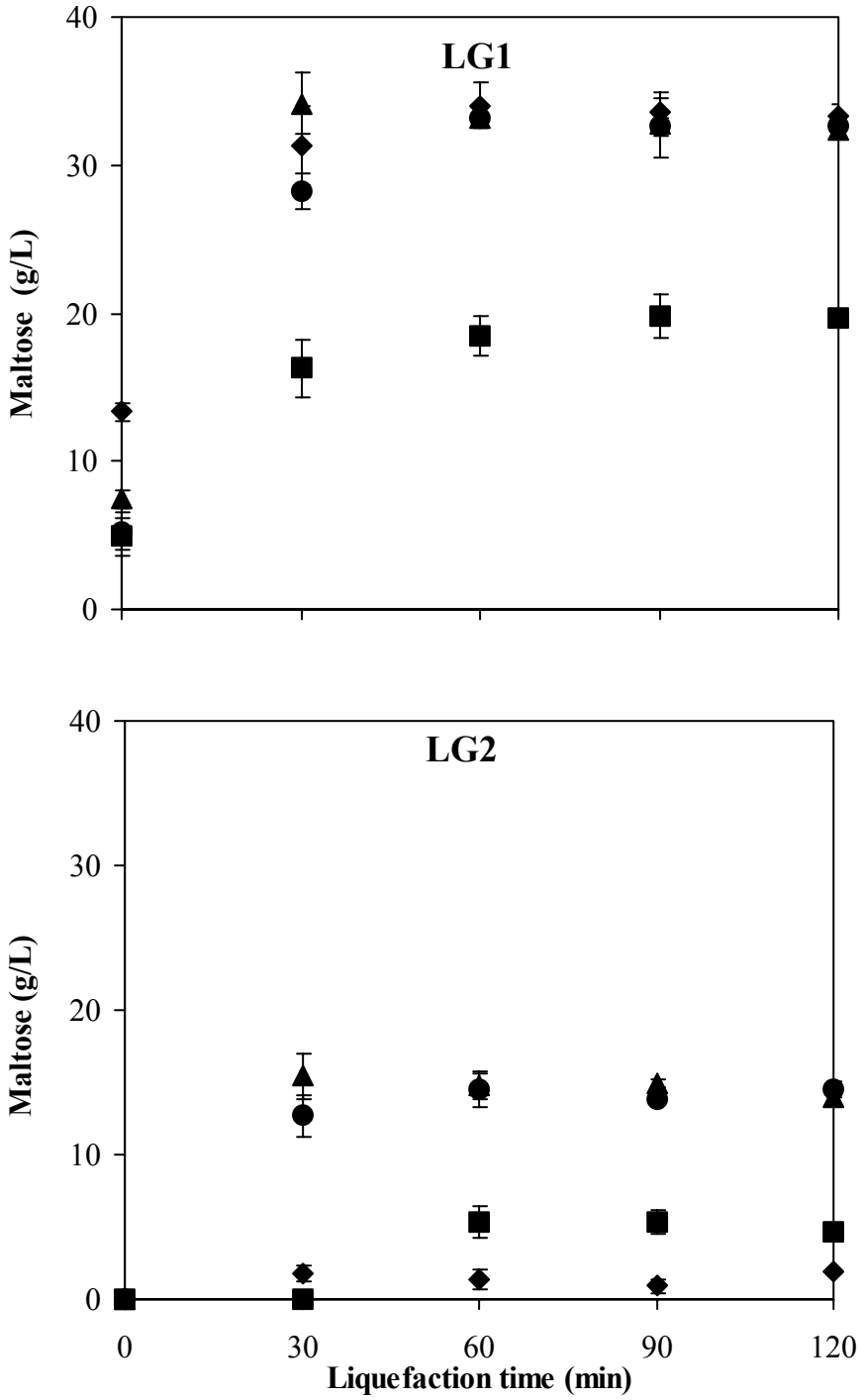


Figure 2.1 Maltose release during liquefaction optimization. Symbols: ◆, 55°C, 200 U/g-flour; ■, 55°C, 100 U/g-flour; ▲, 75°C, 200 U/g-flour; ●, 75°C, 100 U/g-flour (Top: LG1; bottom: LG2). The bars represent the standard deviation (n = 3)

The maltose production after 2 h liquefaction was considerable high in case of LG1. Furthermore, the process conducted at lower temperature (55°C) with higher enzyme activity (200 U/g-flour) resulted in the highest liquefaction yield (0.273 g-maltose/g-flour) (Table 2.2). On the other hand, the liquefaction conducted at 55°C using 100 U/g-flour resulted in the lowest yield (0.019), in case of LG2.

As reported in the literature (Montesinos *et al*, 2000), 2 h liquefaction were absolutely necessary for complete starch hydrolysis, using raw wheat flour as substrate; in that case, a shorter liquefaction time (0.5 or 1 h) brought to a wort with higher viscosity, which did not allow an efficient hydrolysis of glucose polymers.

Table 2.2 Effect of various treatments on the liquefaction yield

Treatment [a]	Liquefaction Yield (Y_L) (g-maltose/g-flour)	
	LG1	LG2
200 U/g; 55°C	0.273 ± 0.017 [b]	0.145 ± 0.002
200 U/g; 75°C	0.249 ± 0.010	0.140 ± 0.005
100 U/g; 55°C	0.200 ± 0.008	0.019 ± 0.002
100 U/g; 75°C	0.148 ± 0.002	0.047 ± 0.006

[a] Two levels of α-amylase (100 or 200 U/g-flour) and temperature (55 or 75°C) were utilized

[b] Mean value ± Standard Deviation (n = 3)

The enzyme thermostable α-amylase (which support well high temperatures without loss on its activity) have been used wisely by various researchers for the liquefaction of wheat flour for ethanol production (Montesinos *et al*, 2000; Favela-Torres *et al*., 1988). Generally, those processes utilize high liquefaction temperatures such as 90-95°C, leading to starch gelatinization during the process; in such cases, the liquefaction can be either conducted in atmospheric batches, pressure batches or

continuous liquefaction, in which crude starch slurries containing as much as 40 % solids (w/w) can be used. In other hand, the commercial α-amylase utilized in this study has an optimum temperature of 65°C (Sigma, 1997), which is relatively low compared to thermostable α-amylase. Thus, the highest liquefaction yield (0.273 g-maltose/g-substrate) obtained by hydrolysis conducted at lower temperature (55°C), might be related to the type of enzyme utilized.

2.3.2 Liquefaction Using α-Amylase or β-Amylase

In order to obtain high ethanol productivities, a key factor is to optimize the amount of maltose available for saccharification, so that releasing more glucose for fermentation; aiming to increase the liquefaction efficiency, β-amylase was used for starch hydrolysis, and its performance was compared to α-amylase. Generally, β-amylase should release higher amounts of maltose from starch hydrolysis, compared to α-amylase (Brautlecht, 1953); that enzyme has the capacity of decomposing into maltose all polysaccharides built up of glucose residues united by α-1,4 glycosidic bonds. Furthermore, this enzyme is commonly used in commercial ethanol processing plants (Mann, 2003).

The flour LG2 was used as substrate and three different levels of enzyme activity were tested: 200, 400 and 800 U/g-flour (Chi *et al.*, 1993). The hydrolysis was conducted up to 4 h, considering the gradual increase in maltose content when 800 U β-amylase/g-flour of were used for liquefaction. The results are presented in Figure 2.2.

Maltose production from LG2 increased proportionally with activity, for both enzymes. Furthermore, α-amylase presented a considerably higher maltose production.

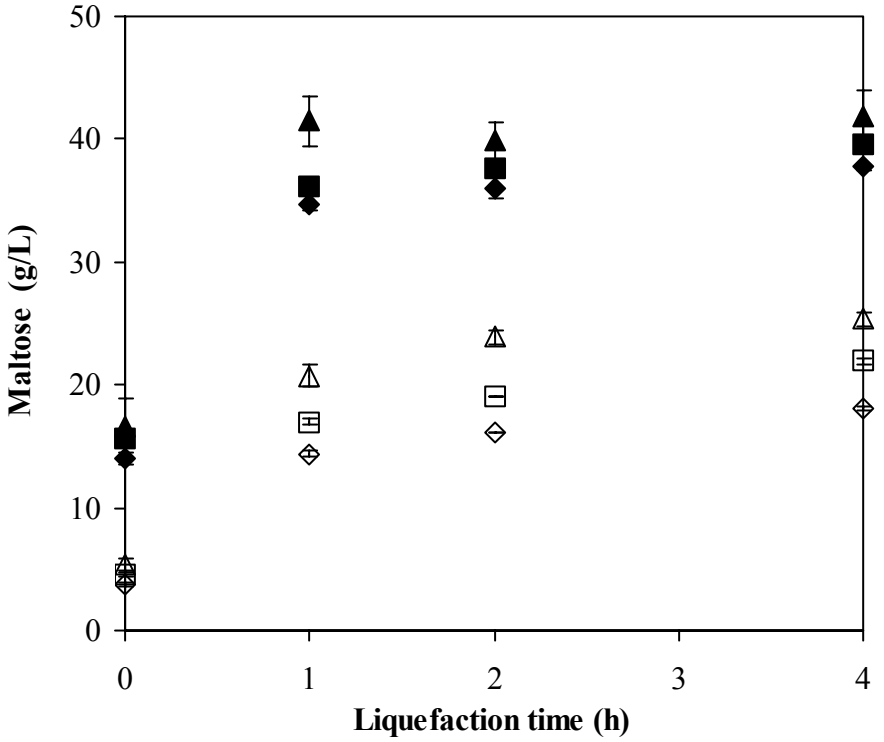


Figure 2.2 Time courses of LG2 liquefaction using: α-amylase (filled), or β-amylase (hollow). Symbols: ◆◇, 200; ■□, 400; ▲△, 800 U/g-flour. The bars represent the standard deviation (n=3)

In view of these results, the process conducted for 2 h at 55°C using 200 U α-amylase/g-flour was selected as the most suitable for LG liquefaction, and used hereafter as a pre-treatment for every fermentation experiments.

2.3.3 Fermentability Test

When LG1 was used as substrate, the ethanol production after 24 h of SSF (38.6 g/L) was notably higher compared to the peak ethanol production from LG 2

(24.9 g/L) obtained after 12 h of SSF, as shown in Figure 2.3, which agrees well with the higher initial starch content in LG (Table 2.1), releasing more fermentable sugars during the liquefaction.

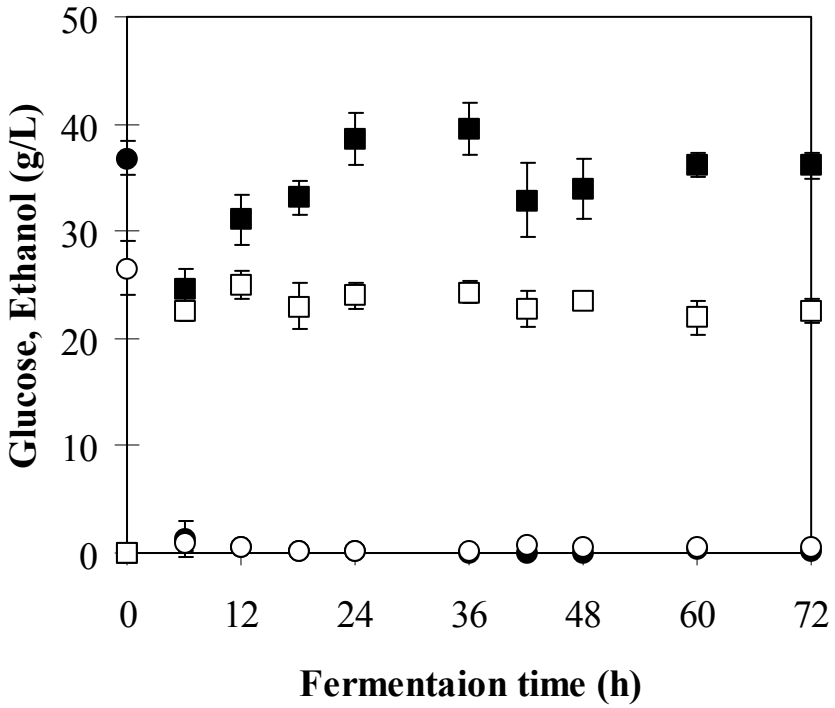


Figure 2.3 Time courses of SSF of LG1 (Filled) and LG2 (hollow). Symbols: ●○, glucose; ■□, ethanol. The bars represent the standard deviation (n=3)

After nearly 6h of SSF the glucose in the fermentation mash was completely consumed, in both cases (using LG1 or LG2 as substrate), remaining nearly constant thereafter.

Sree *et al*. (1999) reported about the ethanol production by SSF of wheat products using *Saccharomyces cerevisiae*. Those authors were able to produce up to 44.2 g-ethanol/L when fine wheat flour was used as substrate, and 34.1 g/L using damaged wheat flour. The amount of ethanol produced from LG1 in this experiment (38.6 g/L) is considerably higher than that obtained from damaged wheat. Once the

starch content in LG1 represents *c.a.* 25% the average starch content in whole-wheat flour (Table 2.1), the final ethanol production from LG1 represented about 87 % of that produced from fine wheat flour (44.2 g/L) by the authors mentioned above.

Lee *et al.* (1992) reported about the ethanol production using *Zymomonas mobilis*. Using slurries containing 100 g/L of sago starch, those authors produced *c.a.* 40 g/L of ethanol, which is nearly the same ethanol production from LG1, in this experiment.

The ethanol yield from LG1 (0.38 g-ethanol/g-flour) was nearly 61 % higher than that obtained from LG2 (Table 2.3). The ethanol yield from LG1 is comparable to the average value from sugarcane (0.39 g-ethanol/g-dry biomass) (Kim *et al*., 2004). Furthermore, the ethanol yield obtained on this study using either substrate (LG1 or LG2) is considerably higher compared to other agricultural crops residues, such as wheat straw (0.23 g/g) or sugarcane bagasse (0.22 g/g) (Daishou, 2004).

Table 2.3 Ethanol yield for different substrates

Substrate	Ethanol Yield ($Y_{P/S}$) (g-ethanol/g-flour)
LG1	0.386 ± 0.024 [a]
LG2	0.240 ± 0.011

[a] Mean value ± Standard Deviation (n = 3)

Taking into account that saccharification occurs simultaneously with fermentation, some glucose should be produced during that process. In this work, the glucose released from starch was promptly used for fermentation, and was rarely detected during the SSF. Various authors have already reported about this early glucose extinction during the SSF, using *e.g.*, soluble starch (Fujii *et al.*, 2001) or raw

cassava starch (Roble, 2003) as substrate and immobilized yeast for fermentation. The nutrient starvation might play an important role in the saccharification performance (Suresh *et al*., 1999).

The difference between the optimum temperature for amyloglucosidase activity (55°C) and yeast growth (35°C) also might play an important role in the process; lower temperatures are preferred because the metabolic activity of the yeast is increased, and this normally results in a faster completion of fermentation (Thomas *et al.*, 1993). An alternative proposed in the literature is to use thermo-tolerant yeast strains, making possible to conduct the fermentation at 42°C with increased ethanol production (Sree *et al*, 1999).

2.3.4 Kinetic Parameters

In order to access the kinetic parameters of dry baker's yeast growth, LG1 was used as substrate for SSF; the results are shown in Figure 2.4. During the first hour of SSF the lag phase was observed, after which the exponential growth phase started. Reaching the stationery phase (assumed to be at *c.a.*8 h of SSF) a slight reduction on cell density was observed, indicating nutrient depletion on the fermentation broth. Further increase in the cell density was observed at the end of the process; this late yeast growth was reported in the literature, and might be related to the ethanol consumption by yeasts at the end of starch saccharification (Fujii *et al*, 2001).

The logarithm number of yeast cells (Log N) was plotted as a function of time (Figure 2.4). The data obtained during the exponential phase were linearized (◆) and correlated well (R^2= 0.976); the slope of the resulting equation (0.1685) was substituted in equation 2.3 to calculate the specific growth rate: $\mu = 0.388\ h^{-1}$.

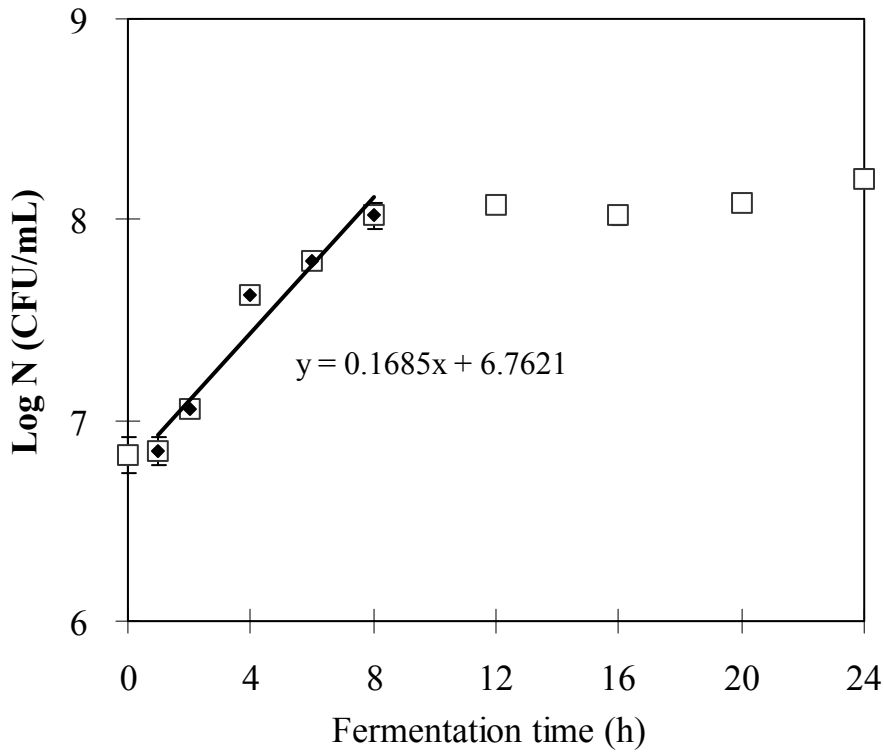


Figure 2.4 - Yeast growth during SSF of LG1. The bars represent the standard deviation (n=3)

The generation time (*g*) for dry baker's yeast was than calculated using equation 2.4, obtaining g = 1.78 h, which is the time required for the population to double the number of cells. This result indicates that the average time required for yeast cells to complete one cell cycle is considerably faster than that of *Zymomonas mobilis* (5.8 h) growing under the same conditions using the same substrate (Neves *et al*., 2005).

Chapter 3

Effect of Substrate Concentration on Ethanol Production by *Zymomonas mobilis*

3.1 Introduction

The progressive expansion of civilization and the related ever-developing science and technology present us with many new problems. It is primarily a question of energy, food and the pollution of our natural environment (Szczodrak *et al*, 1990).

Ethanol is commonly used in a 10% ethanol and 90% gasoline blend (Daishou, 2004). About 90% of all ethanol is derived from sugar or starch crops by fermentation; the rest is produced synthetically. The world ethanol production in 2001 was 31 giga L, and the bulk of the production is located in Brazil and the US, which account for about 62% of world production (Hamelinck *et al*., 2005).

Various agricultural products have been used as raw material for bioethanol production, such as: sugar cane (mainly used in Brazil, India, Thai), sweet corn (US, China), wheat (France, Canada, Sweden), cassava (Thai) among others, and the ethanol yield varies widely, according to the raw material utilized (FIBGE, 2001).

Low-grade wheat flour (LG) is a by-product obtained from wheat milling; the starch content in LG (c.a.15%, Table 2.1) is reduced, if compared to the standard wheat flour (which contains in average 65% starch). The main constituents of LG are the outer parts of kernel, i.e. outer pericarp, aleurone layer and starchy endosperm; it is produced at the tail end of breaks and size reduction system.

In Brazil, the total amount of wheat flour produced in the year 2000 was about 6.8 million ton (FIBGE, 2001), from which about 5 % represent the low-grade wheat flour (Hoseney, 1986).

The objectives of the present study were:

(1) to evaluate the suitability of low-grade wheat flour as substrate for producing bioethanol;
(2) to determine the role of various initial substrate concentration on the performance of *Zymomonas mobilis* during SSF.

3.2 Material and Methods

3.2.1 Raw Material

Low-grade wheat flour (LG), with average moisture content 14%, starch 15.6%, protein 15%, fiber 0.8% and ash 2.7% (Table 2.1); provided by Nisshin Flour Milling Co. Ltd., Japan.

3.2.2 Bacterial Cells and Culture Media

Zymomonas mobilis NBRC 13756 was used as ethanol-producing bacterial strain. The bacterium was maintained in a liquid medium (pH 6.8), which contained (in g/L): glucose, 20; yeast extract, 5 and $MgSO_4 \cdot 7H_2O$, 2, in distilled water.

Cultures were incubated at 30°C for 48 h and then stored at 4°C. Sub-culturing was done every month. This medium was also used for the pre-culture, under the same conditions.

3.2.3 Pre-cultivation

An inoculum containing 10 mL of *Z. mobilis* stock culture was used to inoculate 90 mL of the pre-culture medium in 300 mL erlenmeyer flasks and incubated at 30°C for 24 h, shaken at 100 rpm in aerobic condition. The cell density of starter culture was adjusted in order to obtain approximately $2x10^4$ cells/g-flour, using Optical Density for evaluation of cell density.

3.2.4 Enzymes

Commercial enzymes α-amylase and glucoamylase (as specified in Chapter 2, section 2.2.3), were used for liquefaction and saccharification, respectively.

3.2.5 Liquefaction

LG was used to prepare 1 L-slurries containing 100 g/L, 200 g/L or 300 g-flour/L in distilled water(in separate), and hydrolyzed using 400 U/g-flour of α-amylase, at 55°C for 2h, shaking at 100 rpm.

3.2.6 Simultaneous Saccharification and Fermentation

After liquefaction, the pH of hydrolyzed slurry was adjusted to 4.5, glucoamylase (200 U/g-flour) (Chi *et al*., 1993) and 100 mL of *Z. mobilis* pre-culture (containing *c.a*. $2x10^4$ cells/g-flour) were added for SSF. The mixture was aseptically transferred to a 2-L fermentor (MDL 200 B.E. Marubishi, Japan) (as depicted in Figure 3.1), which was previously sterilized. The fermentor is equipped with temperature and pH automatic controls. NaOH 3M was used to maintain the pH 4.5. The process was conducted at 35°C for 48 h, with mild agitation (100 rpm). Nitrogen gas was bubbled at flow rate 100 mL/min into the reactor, to assure on anaerobic environment.

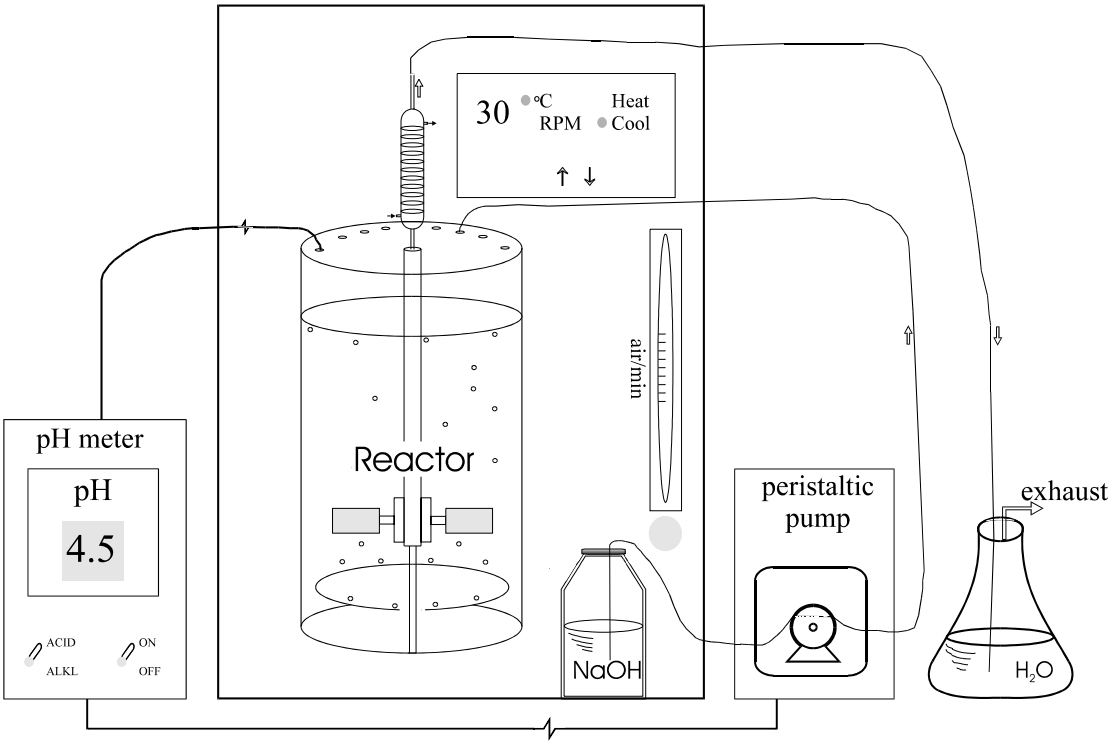


Figure 3.1 Schematic diagram of bioreactor utilized for SSF with pH, temperature, stirrer and gas flow control

3.2.7 Analytical Methods

Glucose, maltose and ethanol concentrations were measured using an HPLC system (Jasco, Japan) as described in the literature (Shiiba *et al*., 1993); all samples were centrifuged at 4,000 rpm for 20 min, and the supernatant was filtered through chromato-disk filters (pore size = 0.45 μm) prior to HPLC analysis. The reducing sugar concentration was determined using the 3,5-DNS method (Bernfeld, 1955). The proximal composition of LG was analyzed using the methodology described in chapter 2 (2.2.4). Viable counts were accessed using the plate count method. Microbial growth rate (μ) was calculated from the time profile growth curve during the exponential growth phase, using the slope method (Barker, 1998) and the generation time (g) was calculated as indicated previously (Equation 2.4).

The ethanol data were analyzed using an exponential law. Experimental values fitted well the model with regression coefficients of more than 0.97. The ethanol productivity (Q)

was calculated by differentiation of experimental ethanol production data (*dP/dt*) at specified time intervals (Jain *et al*., 1985).

The overall volumetric ethanol productivity (Q_v)was calculated relating the final ethanol concentration to the fermentation time.

The ethanol yield ($Y_{P/S}$) (per dry basis of substrate) was calculated as indicated above (Equation 2.2).

3.3 Results and Discussion

Slurries containing different concentrations (*i.e.* 100 g/L, 200 g/L or 300 g/L) of LG were used as substrate for bioethanol production. After the initial liquefaction, the simultaneous saccharification and fermentation process (SSF) was conducted using glucoamylase (for saccharification) and *Z. mobilis* (for fermentation). This bacteria is known for its ability to tolerate high sugar concentrations in the substrate (up to 400 g/L, for some strains), as well as it can tolerate relatively high ethanol concentrations (up to 100 g/L), minimizing the product inhibition. Furthermore, the potential for low biomass yields and high ethanol yields are some of the advantages of using this bacteria for fermentation (Rogers, *et al*., 1982). The results for each run with different substrates are presented below.

3.3.1 Simultaneous Saccharification and Fermentation of Slurries Containing 100 g-flour/L

After nearly 8 h fermentation the glucose level reduced drastically, dropping to zero after a few hours, along with a fast increase in ethanol production, revealing the intimate relationship between these two compounds. After glucose extinction, even though there was a small amount of reducing sugars (others than glucose) in the mash, the ethanol production

completely stagnated, indicating the end of fermentation, probably due to the lack of nutrients, within about 12 h from the start of fermentation (Figure 3.2).

The maltose concentration remained constant (nearly zero) throughout the process, indicating that no more starch was available for hydrolysis after the previous liquefaction.

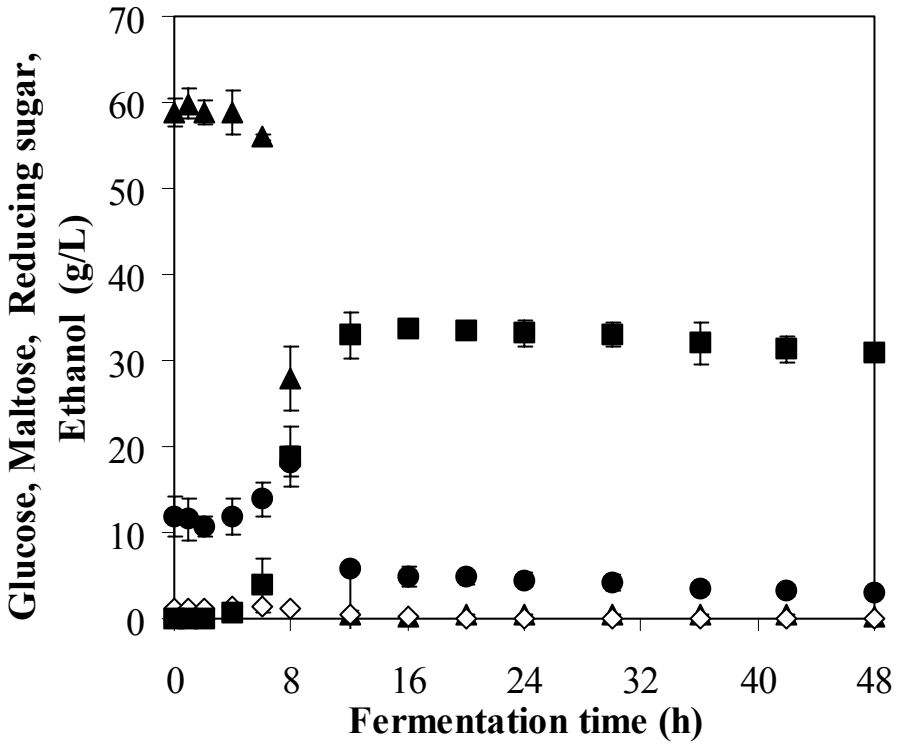


Figure 3.2 SSF of Low-grade flour. Initial substrate concentration: 100 g/L. Symbols: ▲, Glucose; ◇, Maltose; ●, Reducing sugars; ■, Ethanol. The bars represent the standard deviation (n=2)

Despite the peak ethanol concentration was relatively low (c.a. 33.6 g/L), the ethanol yield in case of substrates containing 100 g/L was the highest (0.188 g/g-substrate) among the three substrates tested (Table 3.1).

3.3.2 Simultaneous Saccharification and Fermentation of Slurries Containing 200 g-flour/L

Once again the glucose depletion was observed at the fermentation onset, as it was it was previously observed for slurries containing 100 g/L, though a small peak in glucose concentration indicated that the saccharification had occurred.

Starting with 200 g-substrate/L provided more fermentable sugars to the microorganisms, as indicated in Figure 3.3 by a gradual decrease in reducing sugars concentration throughout the process, nevertheless the glucose exhaustion, ensuring that ethanol production continued for a few more hours in absence of glucose, until a peak ethanol concentration (c.a. 51.5 g/L) was obtained after 24 h of fermentation.

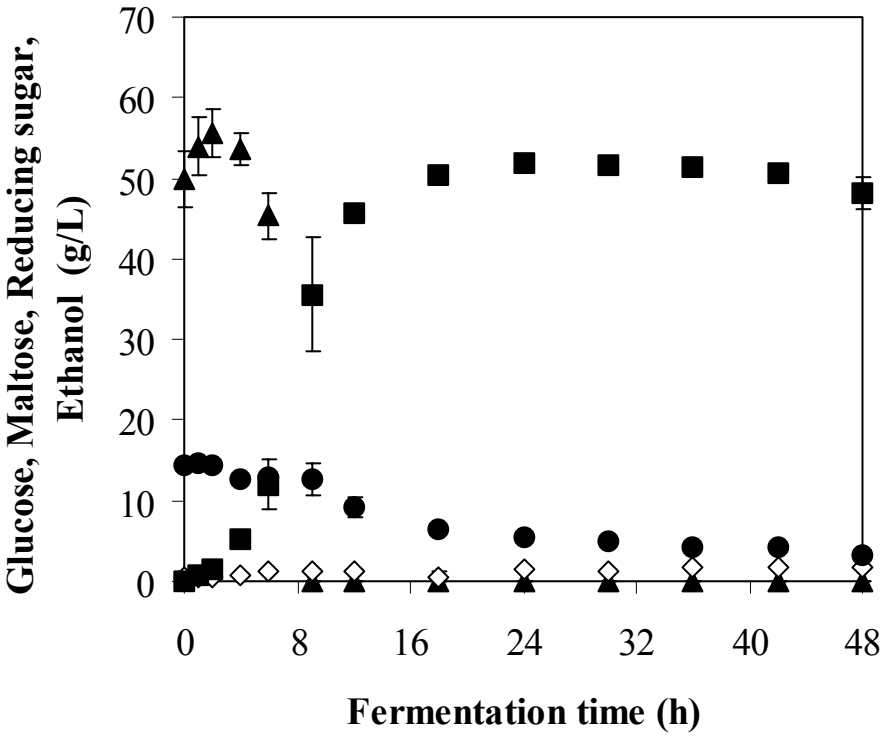


Figure 3.3 SSF of Low-grade flour. Initial substrate concentration: 200 g/L. Symbols: ▲, Glucose; ◇, Maltose; ●, Reducing sugars; ■, Ethanol. The bars represent the standard deviation (n=2)

Increasing the initial substrate concentration up to 200 g/L resulted in a higher ethanol production, without denoting any counter effects, neither on ethanol production nor on microbial growth (Figure 3.5).

As previously observed for substrates containing 100 g/L, in this experiment there was no considerable maltose production, indicating that the liquefaction had been complete leaving no residual starch for hydrolysis.

Considering that ethanol yield is related to the mass of initial substrate (in dry basis), this might explain the reason why the ethanol yield obtained from substrates containing 200 g/L (0.26 g/g-substrate) was lower than that from slurries containing 100 g/L (0.33 g/g), despite the higher ethanol concentration.

3.3.3 Simultaneous Saccharification and Fermentation of Slurries Containing 300 g-flour/L

Further increase at the initial substrate concentration resulted in noticeable suppression of both enzymatic and bacterial activity. Likely, *Z. mobilis* was not able to assimilate the glucose from the fermentation mash, due to glucose saturation, as was the case with slurries containing 100g/L or 200 g/L.

In case of using slurries containing 300 g/L, the presence of considerable amounts of maltose during the process onset indicated that the starch hydrolysis had been incomplete during the previous liquefaction (Figure 3.4). For the sake of comparison, the same process variables were utilized in all experiments, e.g. hydrolysis time, temperature and agitation. As for slurries with an initial flour concentration higher than 200 g/L, prolonged liquefaction times should be tested as well, in order to ensure the complete starch hydrolysis, which means to obtain the maximum amount of fermentable sugars, before starting the fermentation.

As the glucose consumption was much lower, compared to other substrates (e.g. glucose depletion was not observed in this case), it is reasonable to assume that other

fermentable sugars were consumed as main carbon source for ethanol production; this fact is supported by the reducing sugars pattern, indicating the presence of other sugars in the medium, after maltose extinction.

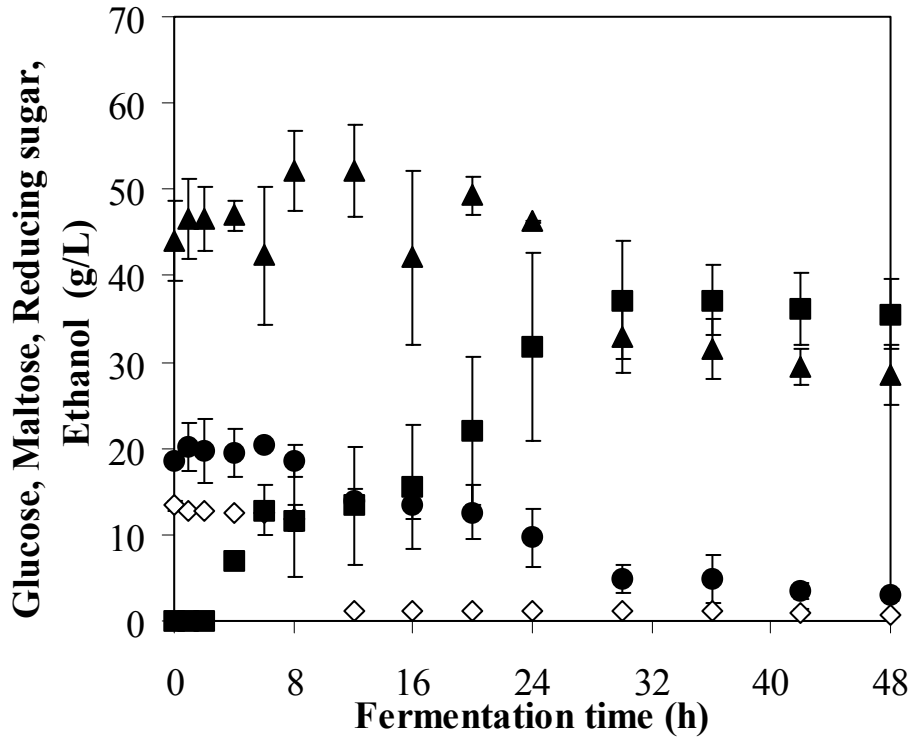


Figure 3.4 SSF of Low-grade flour. Initial substrate concentration: 300 g/L. Symbols: ▲, Glucose; ◇, Maltose; ●, Reducing sugars; ■, Ethanol. The bars represent the standard deviation (n=2)

The peak ethanol obtained using substrates containing 300 g-flour/L was *c.a.* 37.2 g/L, meanwhile the ethanol yield was the lowest (0.13 g/g-substrate) among the three different substrates tested.

The high solids content (*c.a.* 300 g/L) might have affected the particles diffusion into the fermentation mash, due to physical limitations (difficulty to shake the medium evenly, pipes obstruction, among others). Thus, the data distribution was not normal, and the experimental reproducibility of was considerably low, compared to other substrate concentrations (depicted by the errors bars in Figures 3.2, 3.3 and 3.4).

Furthermore, the high particle content (in case of 300 g/L) may have caused non-uniform nutrients distribution in the fermentation mash, likely due to saturation of the medium, making difficult the diffusion of water molecules into the material structure as well as lowering the water activity. As a result, the fermentation process was delayed, taking a considerable amount of time to increase the ethanol concentration, compared to the substrate containing 200 g/L (Figure 3.3); the fermentation efficiency was also affected, as indicated by the final ethanol concentration (*c.a.* 35 g/L), which is lower than that obtained from the substrate containing 200 g/L (48 g/L).

Utilizing molasses with high sugar concentration some authors (Ueno *et* al., 2003) were able to increase the yield on lactic acid fermentation by varying enzyme concentration and hydrolysis time, converting polysaccharides into monosaccharides, which are more easily assimilated by bacteria. Increasing the amylase concentration during liquefaction, as well as the hydrolysis time, might increase the ethanol yield when utilizing high flour concentration in the slurry.

3.3.4 Bacterial Growth During Fermentation Using Various Substrate Concentration

Increasing the substrate concentration up to 200 g/L resulted in a considerable increase in cell density on the fermentation mash, along with a slight increase in microbial growth rate, resulting in faster generation time (Table 3.1).

Due to the unease to assimilate the carbon source in the fermentation mash containing high substrate concentration (300 g/L), the bacterial growth was delayed along with a relatively prolonged stationary phase (until *c.a.* 24 h fermentation), followed by a stiff increase in cell density, revealing that the nutrients consumed by that time were mostly converted to biomass, rather than being used for ethanol production (Figure3.5).

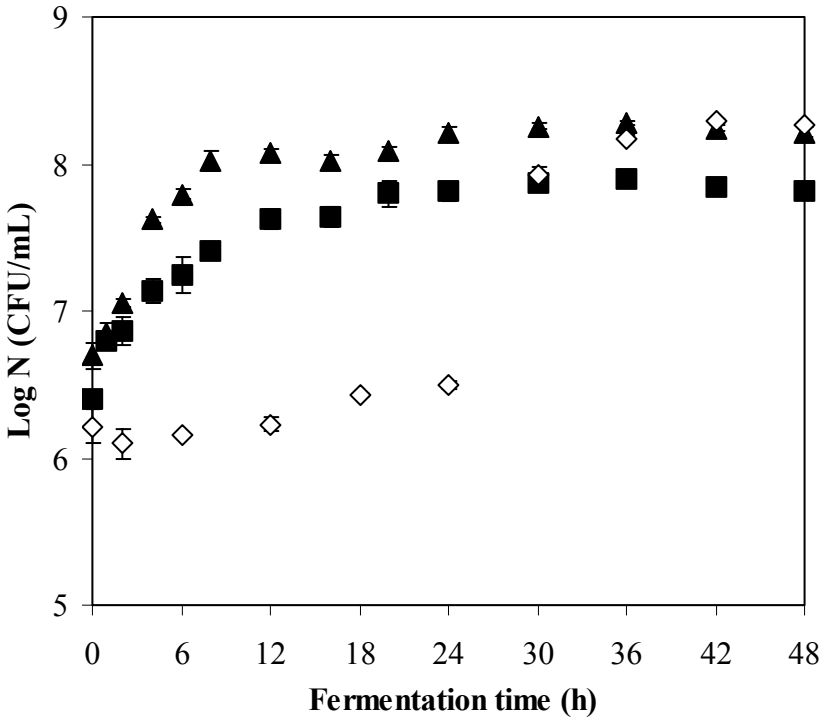


Figure 3.5 *Zymomonas mobilis* microbial growth from LG at various initial substrate concentration. Symbols: ■, 100 g/L; ▲, 200 g/L; ◇, 300 g/L. The bars represent the standard deviation (n=3)

Should biomass production be the scope of this work, the substrate containing 300 g/L of low-grade flour would be the most indicated, even thought the process might take longer compared to other substrates, once *Z. mobilis* enters the exponential growth phase (after *c.a.* 24 h) the cell density might increase vigorously.

3.3.5 Kinetics of Simultaneous Saccharification and Fermentation Using Various Levels of Low-grade Wheat Flour

Table 3.1 shows some kinetic parameters related to the microbial growth and ethanol production during SSF of low-grade flour by *Z. mobilis*. The peak overall volumetric ethanol productivity (Q_v) (2.57 g-ethanol/L·h) was obtained when the substrate containing 200 g/L

was utilized, followed by the slurries containing 100 g/L and 300 g /L (1.4 g/L·h and 0.93 g/L·h, respectively).

Table 3.1 Kinetic parameters during fermentation

Kinetic parameter	Substrate concentration (g /L)		
	100	200	300
Time intervals for calculation (h)	0-24	0-20	0-40
Growth rate (μ), (h^{-1})	0.056	0.058	0.132
Generation time (g), (h)	12.332	11.941	5.261
Ethanol concentration (P), (g/L) [a]	33.6 ± 2.3 [b]	51.5 ± 1.7	37.2 ± 3.9
Ethanol yield ($Y_{P/S}$), (g/g-substrate)	0.33 ± 0.07	0.26 ± 0.01	0.13 ± 0.04
Overall volumetric ethanol productivity (Q_v), (g/L·h)	1.40 ± 0.04	2.57 ± 0.16	0.93 ± 0.08

[a] Peak ethanol concentration

[b] Mean value ± Standard Deviation (n = 2)

In other hand, the maximum ethanol yield (0.33 g/g-substrate) was obtained from the substrate containing 100 g/L, followed by the substrate containing 200 g/L (0.26 g/g-substrate). The ethanol yield was quite low in case of the substrate containing 300 g/L, as observed with the peak ethanol production, suggesting the unease of *Zymomonas mobilis* to assimilate nutrients, when growing in presence of high substrate concentration.

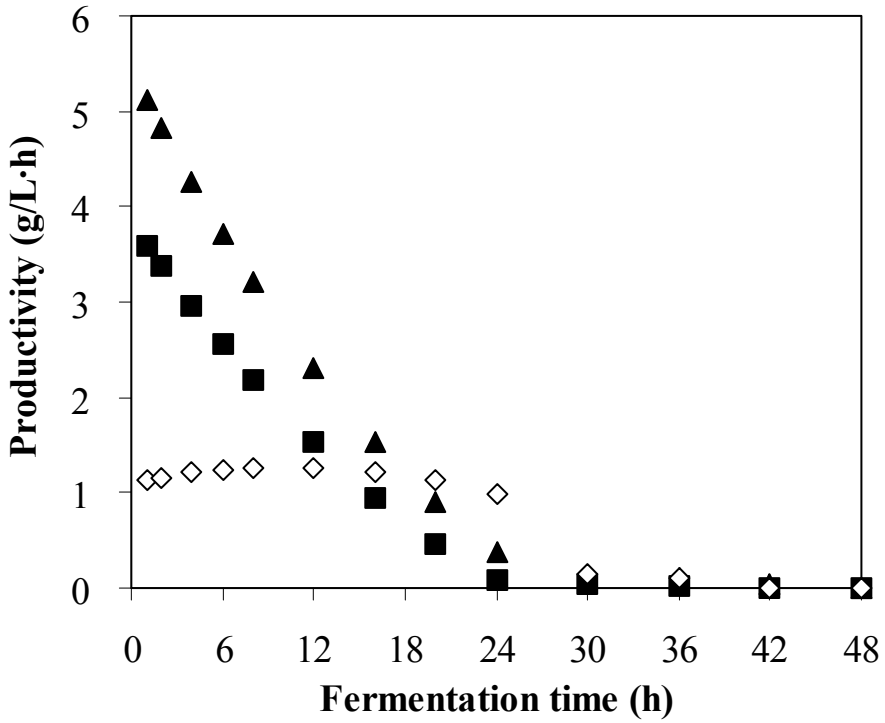


Figure 3.6 Ethanol productivity using *Zymomonas mobilis* and LG at various concentration. Symbols: ■, 100 g/L; ▲, 200 g/L; ◇, 300 g/L.

When the intermediate initial substrate concentration (200 g/L) was utilized, a peak ethanol concentration (51.5 g/L) was obtained after 30 h fermentation, with an average ethanol yield of *c.a.* 0.26 g/g-substrate, which is nearly 4..3 fold the average ethanol yield from sugar cane (Moreira and Goldemberg, 1999), and slightly higher than the yield from cassava (Barrets de Menezes, 1982). The ethanol yield from LG represents *c.a.* 73% of the yield obtained from sound wheat grains, as mentioned in the literature (Suresh, *et al*., 1999).

Chapter 4

Fermentation of Low-grade Wheat Flour

4.1 Introduction

Traditionally hydrolysis and fermentation processes are done in separate steps using either a single reactor or a number of reactors in series (Gorinstein, 1993). The hydrolysis step can be done with either purified enzymes, crude enzymes or microorganisms (Roble, 2003).

The combination of saccharification and fermentation in a single step, so-called simultaneous saccharification and fermentation, was reported to increase the alcohol yield and reduce the residual starch content in the fermentation by-product (Olsen, 2001; Montesinos, 2000; Montesinos, 2000b).

The objectives of this study were:

(1) To conduct the saccharification and fermentation in separate or simultaneously.

(2) To produce α-amylase in the laboratory and compare to the commercial enzyme.

4.2 Separate Hydrolysis and Fermentation of Low-grade Wheat Flour Using *S. cerevisiae*

In this study the starch containing substrates were initially hydrolyzed by the action of amylolytic enzymes: α-amylase (for liquefaction) and glucoamylase (for saccharification). After complete hydrolysis, the fermentation was conducted in separate as a single step.

4.2.1 Material and Methods

The main stages and experimental conditions utilized during the SHF are briefly described in Figure 4.1.

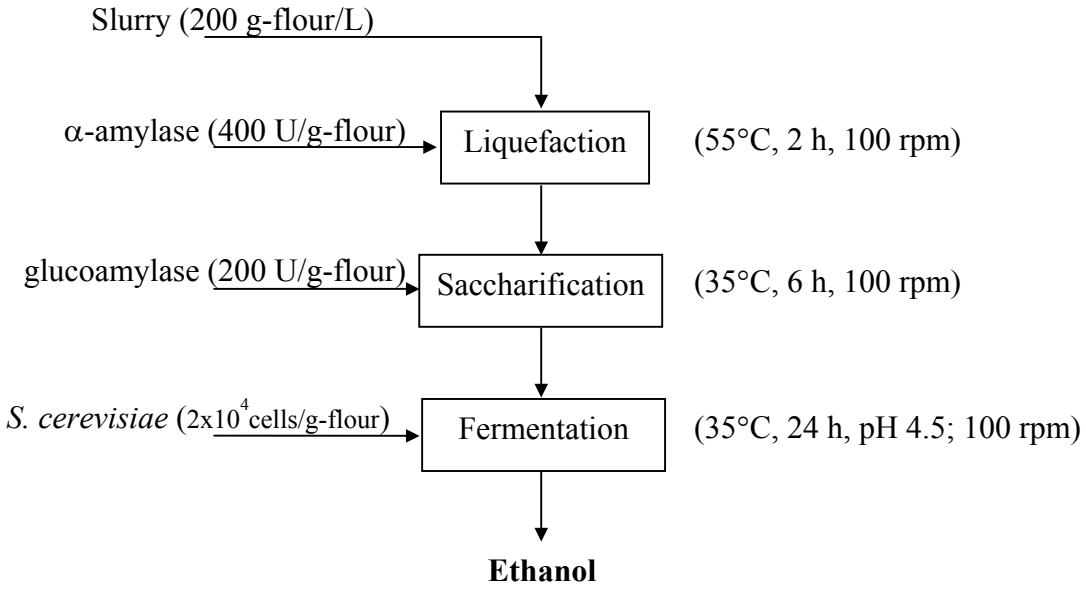


Figure 4.1 Alcohol production by SHF: Main process stages

4.2.1.1 Raw Material

Low-grade wheat flour (LG) with average moisture content 14% (w/w), starch 15.6%, protein 15%, fiber 0.8% and ash 2.7%; provided by Nisshin Flour Milling Co. Ltd., Japan.

4.2.1.2 Microorganisms and Culture Media

Saccharomyces cerevisiae NBRC 2114 was used as ethanol-producing yeast strain. It was maintained in a liquid medium (pH 5.6), which contained (in g/L): glucose, 10; peptone, 5; yeast extract, 3 and malt extract, 3, in distilled water. Cultures were incubated at 28°C for 24 h and then stored at 4°C. Sub-culturing was done every month. This medium was also used for the pre-culture, under the same conditions.

4.2.1.3 Pre-cultivation

An inoculum containing 10 mL of *S. cerevisiae* stock culture was used to inoculate 90 mL of the pre-culture medium in 300 mL erlenmeyer flasks and incubated at 28°C for 16 h, shaken in aerobic condition. The cell density of starter culture was adjusted in order to obtain approximately $2x10^4$ cells/g-flour, using Optical Density for evaluation of cell density.

4.2.1.4 Enzymes

Commercial enzymes α-amylase and glucoamylase (as specified in Chapter 2, section 2.2.3), were used for liquefaction and saccharification, respectively.

4.2.1.5 Liquefaction

LG was used to prepare 1 L slurries containing 200 g-flour/L in distilled water, and hydrolyzed using 400 U α-amylase/g-flour, at 55°C for 2h, shaking at 100 rpm.

4.2.1.6 Saccharification

The enzyme glucoamylase was added (200 U/g-flour) to the liquefied slurry, and the saccharification was conducted in 1 L erlenmeyer flasks at 35°C for 6 h, shaking at 100 rpm.

4.2.1.7 Single Fermentation

After saccharification the pH of saccharified slurry was adjusted to 4.5, and 100 mL of *S. cerevisiae* pre-culture were added to the mash. The fermentation was conducted in a 2 L fermentor (MDL 200 B.E. Marubishi, Japan) at 35°C for 24 h, with mild agitation (100 rpm). Nitrogen gas was bubbled into the reactor at a flow rate 100 mL/min, to assure on anaerobic environment.

4.2.1.8 Analytical Methods

Glucose, maltose, ethanol and reducing sugar were evaluated as previously described (Chapter 3, section 3.2.7). The LG proximal composition was analyzed as described in chapter 2 (section 2.2.4). Viable counts were accessed using plate count method.

4.2.2 Results and Discussion

Slurries containing 200 g/L were hydrolyzed and subsequently fermented by SHF process, obtaining the maximum ethanol production (116 g/L) after 24 h of fermentation (Figure 4.2).

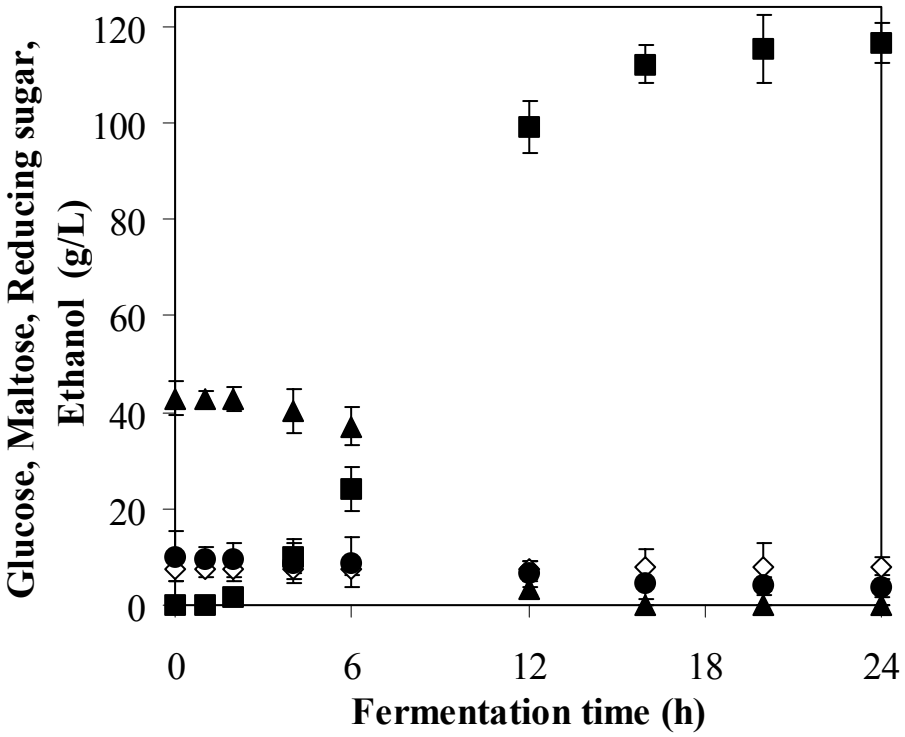


Figure 4.2 Time courses of low-grade flour conversion to ethanol by SHF. Symbols: ▲, Glucose; ◇, Maltose; ●, Reducing sugars; ■, Ethanol. The bars represent the standard deviation (n=3)

The high ethanol concentration obtained might have inhibited the yeast, which generally support well ethanol concentrations up to 100 g/L (Klass, 1981; Saha, 1997); above this concentration, ethanol may induce to product inhibition, resulting in incomplete sugar consumption.

Conducting the saccharification previous to fermentation resulted in direct relation between glucose consumption and ethanol production throughout the SHF, as indicated in Figure 4.3. The glucose consumption (%) was expressed as the glucose concentration at the sampling time related to the initial glucose concentration (g/L).

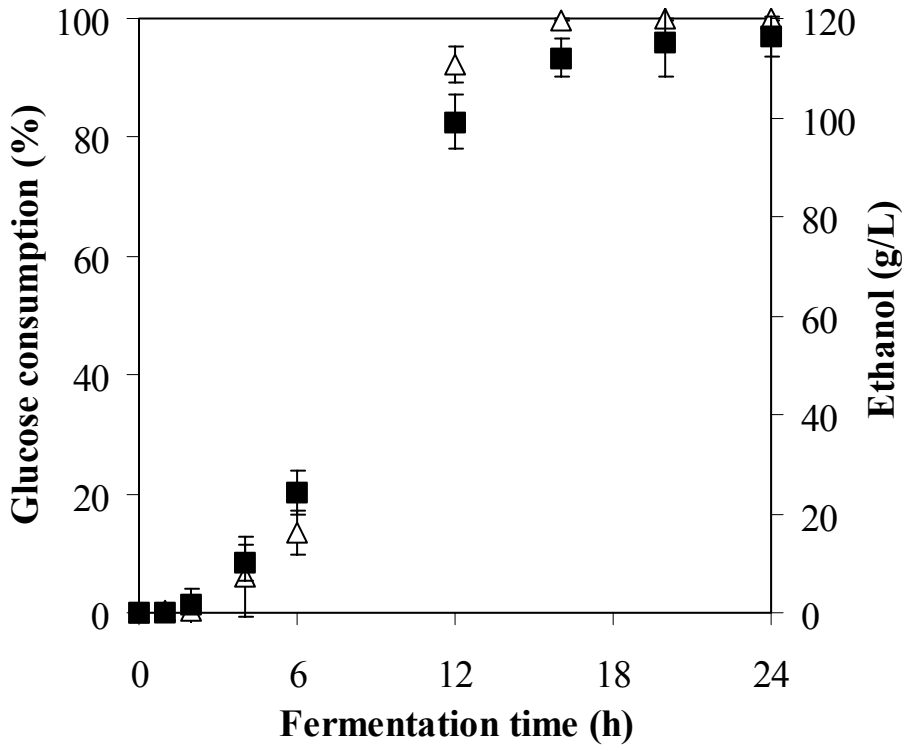


Figure 4.3 Relation between glucose consumption and ethanol production during the SHF. Symbols: △, Glucose consumption; ■, Ethanol. The bars represent the standard deviation (n=3)

From the results above, the ethanol production (M_{pe}) was assumed to be exclusively dependent on the glucose consumed (M_{cg}) from the fermentation mash, with a nearly linear relation (R^2= 0.996), and could be expressed by equation 4.1.

$$M_{pe} = 2.410 + 1.113\, M_{cg} \qquad (4.1)$$

A direct dependency of ethanol production on glucose consumption during fermentation was previously observed by Borzani *et* al. (1998), utilizing sugar-cane blackstrap molasses as the carbon source. In that case the major sugar source (sucrose) was converted to glucose by invertase, which is naturally produced by the yeast, emphasizing the needless of enzymatic hydrolysis prior to fermentation. Thus, the sugars initially present in the fermentation mash were directly converted into ethanol.

4.3 Simultaneous Saccharification and Fermentation of Low-grade Wheat Flour Using *S. cerevisiae*

In this process the saccharification of sugars released during starch hydrolysis (mainly maltose) is conducted simultaneously with fermentation. Immediately after liquefaction by α-amylase, the enzyme glucoamylase was added concomitantly with yeasts to the slurry and the SSF was conducted in a single reactor.

4.3.1 Materials and Methods

The raw material, microorganisms, media and enzymes utilized for SSF were the same as described previously for the SHF process (section 4.2.1). The liquefaction process was conducted as previously described (4.2.1.5).

The main stages and experimental conditions utilized during the SSF are briefly described in Figure 4.4.

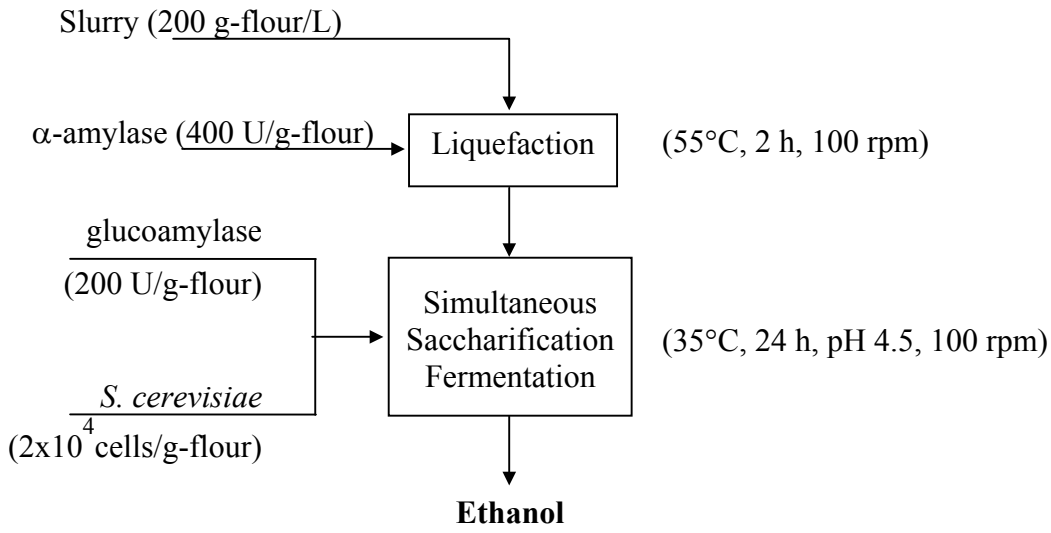


Figure 4.4 Alcohol production by SSF: Main process stages

4.3.1.1 Simultaneous Saccharification and Fermentation

After liquefaction the pH of hydrolyzed slurry was adjusted to 4.5, glucoamylase (200 U/g-flour) and 100 mL of *S. cerevisiae* pre-culture (containing *c.a.* 2x10^4 cells/g-flour) were added to the mash. The mixture was aseptically transferred to a 2 L fermentor (MDL 200 B.E. Marubishi, Japan), and the SSF was conducted under the experimental conditions previously described in chapter 3 (section 3.2.6).

4.3.1.2 Analytical Methods

Glucose, maltose, ethanol, reducing sugar and viable counts were evaluated as described in chapter 3 (section 3.2.7).

4.3.2 Results and Discussion

The ethanol production by SSF of LG using *S. cerevisiae* was considerably lower than that obtained by SHF, as indicated in Figure 4.5.

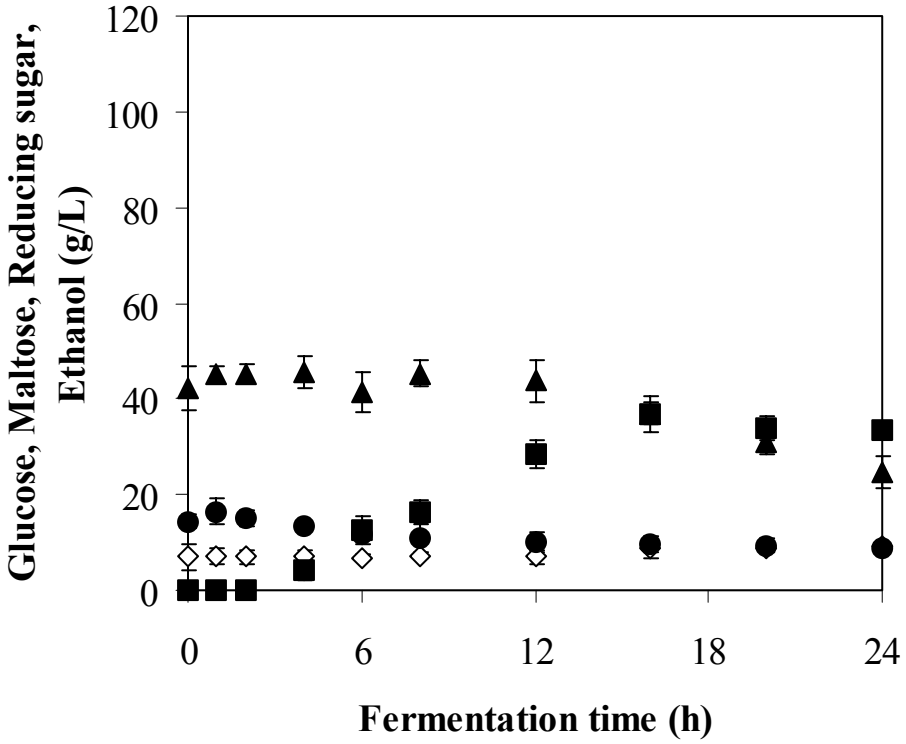


Figure 4.5 Time courses of low-grade flour conversion to ethanol by SSF. Symbols: ▲, Glucose; ◇, Maltose; ●, Reducing sugars; ■, Ethanol. The bars represent the standard deviation (n=3)

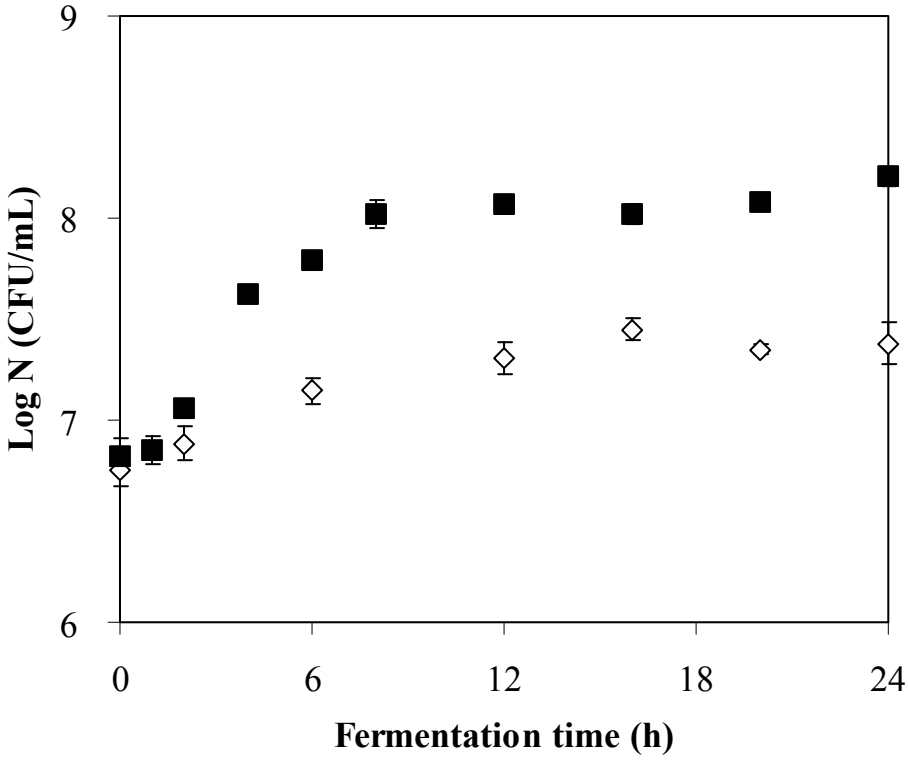


Figure 4.6 Effect of fermentation process on microbial growth. Symbols: ◇, SHF; ■, SSF. The bars represent the standard deviation (n=3)

The microbial growth pattern of *S. cerevisiae* during the fermentation of LG by different processes (SHF and SSF) is depicted in Figure 4.6.

The SSF process showed a considerably higher cell density. Furthermore, during the simultaneous process the yeast cells grew faster.

One disadvantage of conducting the saccharification and fermentation processes simultaneously is that the optimal conditions (especially temperature and pH) for enzymatic hydrolysis generally differ from those for fermentation. The optimum temperature for glucoamylase is *c.a.* 55°C, which is considerably higher than that for yeasts (Roble, 2003b).

4.4 α-Amylase Production Using *Bacillus subtilis*, Followed by Simultaneous Saccharification and Fermentation of Low-grade Wheat Flour Using *S. cerevisiae*

In this study the amylolytic enzyme α-amylase was produced using *B. subtilis*, and its performance was compared to the commercial α-amylase utilized at the previous studies SHF and SSF (sections 4.2 and 4.3). After liquefaction, commercial glucoamylase was added concomitantly with *S. cerevisiae* starter culture, and the SSF was conducted as indicated earlier in this chapter (section 4.3.1.1).

The main stages and experimental conditions utilized during *α*-Amylase production and posterior SSF of LG are briefly described in Figure 4.7.

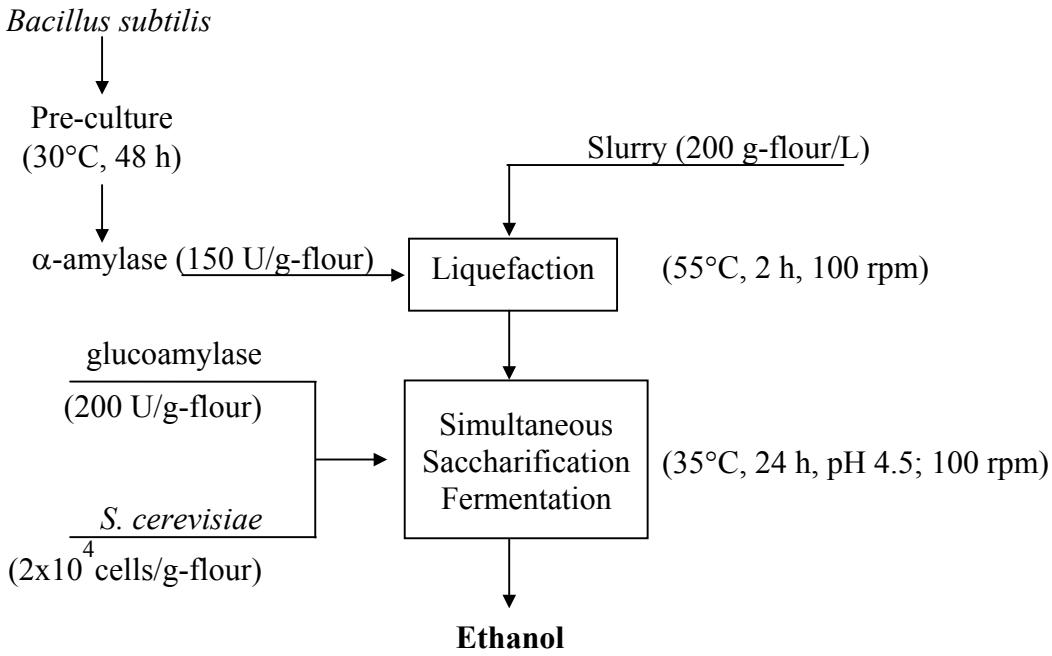


Figure 4.7 Alcohol production by SSF using α-amylase from *Bacillus subtilis*: Main process stages

4.4.1 Materials and Methods

4.4.1.1 Raw Material

Low-grade wheat flour (LG) provided by Nisshin Flour Milling Co. Ltd., Japan. The average composition of LG was described earlier in this chapter (section 4.2.1.1).

4.4.1.2 Microorganisms and Culture Media

Bacillus subtilis subsp. *subtilis* NBRC 3134 was used as α-amylase producing bacteria. The bacterium was maintained in a liquid medium (pH 7.0) which contained (in g/L): polypepton, 10; yeast extract, 2 and $MgSO_4 \cdot 7H_2O$, 1, in distilled water. Cultures were incubated at 30°C for 24 h and then stored at 4°C. This medium was also used for α-amylase production.

Saccharomyces cerevisiae NBRC 2114 was used as ethanol-producing yeast strain. It was maintained in a liquid medium (pH 5.6) which contained (in g/L): glucose, 10; peptone, 5; yeast extract, 3 and malt extract, 3, in distilled water. Cultures were incubated at 28°C for 24 h and then stored at 4°C. This medium was also used for the pre-culture, under the same conditions. Sub-culturing of both microorganisms was done every month.

4.4.1.3 Pre-cultivation of *Bacillus subtilis* and α-Amylase Production

An inoculum containing 10 mL of the stock culture was used to inoculate 90 mL of the pre-culture medium in 300 mL erlenmeyer flasks and incubated at 30°C for 48 h, shaken at 100 rpm in aerobic condition.

Native *B. subtilis* is known to express genes encoding starch-hydrolysing enzymes, such as α-amylase activity (Moraes, *et al*., 1995). Thus, the pre-culture of *B. subtilis* was used as enzyme preparation for amylase activity assay.

4.4.1.4 α-Amylase Assay

The *B. subtilis* pre-culture was centrifuged at 4,000 rpm for 30 min, and the supernatant was used to evaluate the enzyme activity, which was determined using the iodine method (Thomsen, 1983). The substrate for α-amylase reaction was prepared by adding 0.2 g soluble starch to 100 mL boiling 50 mM sodium acetate buffer (pH 5.9) and the solution was cooled to 40°C. The iodine reagent was made by diluting 1 mL stock solution (0.5 % (w/v) I_2 in 5 % KI) in 500 mL deionised water containing 5 mL of 5 M HCl.

The assay consisted of incubating 200 μL enzyme solution with 1 mL of starch solution at 40°C for 10 min. To stop the reaction, an aliquot (200 μL) was

added to 5 mL iodine solution and the starch degradation was measured using a spectrophotometer set at 620 nm, against an appropriate blank. One unit of α-amylase activity was defined as the quantity of enzyme required to hydrolyse 0.1 mg starch in 10 min. at 40°C, when 2.0 mg starch was present at the start of the reaction.

Using the iodine method, a calibration curve for OD_{620} was prepared (0.2, 0.4, 0.6 and 0.8 mg-starch/mL; R^2 = 0.9903) to evaluate the starch degradation by the enzyme preparation. After 10 min of enzymatic hydrolysis the residual starch was 0.04 mg; taking into account the initial amount of starch (2.0 mg), 200 μL of enzyme preparation degraded 1.96 mg starch, which leads to an approximate α-amylase activity of 98 U/mL.

4.4.1.5 Enzymes

α-Amylase from *Bacillus subtilis* produced in this laboratory (98 U/mL), and commercial glucoamylase (as specified in Chapter 2, section 2.2.3), were used for liquefaction and saccharification, respectively.

4.4.1.6 Liquefaction

An aliquote containing 300 mL of *B. subtilis* pre-culture was inoculated into LG slurries previously prepared in distilled water, in order to obtain a final slurry concentration of 200 g-flour/L; the enzymatic load used for liquefaction was *c.a.* 150 U α-amylase/g-flour. The hydrolysis was conducted at 55°C for 2h shaking at 100 rpm.

4.4.1.7 Simultaneous Saccharification and Fermentation

After liquefaction, the pH of hydrolyzed slurry was adjusted to 4.5, glucoamylase (200 U/g-flour) and 100 mL of *S. cerevisiae* pre-culture (containing *c.a.*

2x10^4 cells/g-flour) were added to the mash. The mixture was aseptically transferred to a 2-L fermentor (MDL 200 B.E. Marubishi, Japan), and the SSF was conducted under the experimental conditions previously described in chapter 3 (section 3.2.6).

4.4.1.8 Analytical Methods

Glucose, maltose, ethanol, reducing sugar and viable counts were evaluated as previously described in chapter 3 (section 3.2.7).

4.4.2 Results and Discussion

The results of LG conversion to ethanol using α-amylase produced by *B. subtilis* followed by SSF are shown in Figure 4.8.

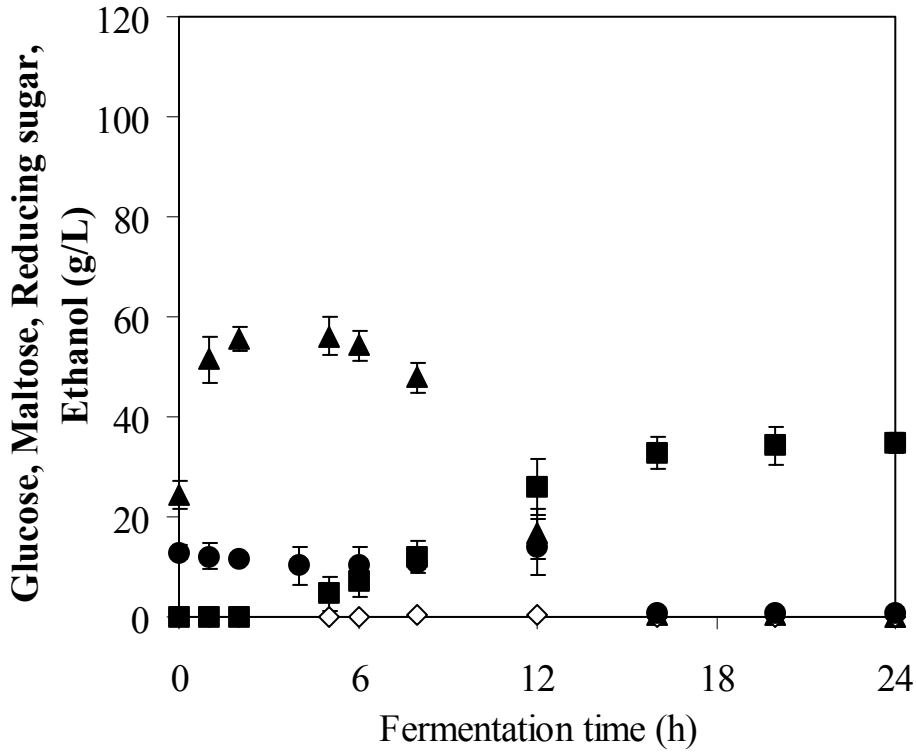


Figure 4.8 Time course of LG conversion to ethanol by SSF using α-amylase from *Bacillus subtilis*. Symbols: ▲, Glucose; ◇, Maltose; ●, Reducing sugars; ■, Ethanol. The bars represent the standard deviation (n=3)

Compared to the commercial enzyme (Figure 4.5), the α-amylase produced from *B. subtilis* revealed inferior performance during starch hydrolysis, resulting in nearly undetectable maltose throughout the SSF process. In other hand, the glucose release during the SSF using the enzyme preparation was considerably higher, along with a slightly higher ethanol production (34.6 g/L) compared to the commercial enzyme (33.3 g/L).

The cultivation process used for enzyme production might play a major role in biological processes. For instance, continuous enzyme production is known to lead to higher productivities compared to fed-batch or batch processes. Klass *et al.* (1981) reported about β-glucosidase production by *T. reesei* using batch (from 96 h up to 120 h) or continuous processes (residence time of 50 h). The resulting enzyme activity as well as the amount of enzyme itself, expressed by the protein content in the fermentation medium, were considerably higher when cultivated under continuous process. In this work α-amylase was produced by *B. subtilis* using small-scale batch processes, which might have led to the low enzyme activity obtained.

In this study a liquid culture system was utilized, with both microorganisms *B. subtilis* and *S. cerevisiae* suspended in the fermentation mash. Thus, substrate competition between those microorganisms may be a reasonable explanation for the low fermentation efficiency. Many studies about the use of mixed culture have been published emphasizing the increased ethanol yield and productivities obtained in those systems compared to single culture processes. One possible alternative to avoid the competition between microorganisms in mixed culture systems is to immobilize them using different carriers, such as gel beads (Ogbonna *et al*., 1991) or loofa sponge (Ogbonna *et al*., 2001; Roble *et al*., 2003). Many advantages can be reached using coimmobilized mixed culture systems having the abilities of two different

microorganisms simultaneously, *e.g.* using gel beads as carrier, aerobic and anaerobic microorganisms spontaneously exhibit "habitat segregation" on the surface and in the center of the gel beads (Tanaka *et al*, 1986).

As an attempt to reduce the cost of the process, nutrient broth, soluble starch or other sources of carbon or nitrogen, which are essential for the enzyme production and are very expensive, may be replaced with more economically available agricultural by-products. Low-grade flour can be used as well for that purpose, taking into account its starch and protein content (*c.a.* 10-15% and 15-16%, respectively; Table 1). Moreover, it is available at much lower prices, compared to other components normally used as fermentation medium.

Chapter 5

Fermentation Performance of Various Wheat Products

5.1 Introduction

With the search for alternative renewable energy sources, biofuels are becoming a viable solution, as they are non-fossil fuels from a renewable agricultural source. Of all biofuels, ethanol has been trusted as an alternate fuel for the future and is already produced on a fair scale (about 14-26 million tons) worldwide. The bulk of the production is located in Brazil (14 billion L produced in 2003), and the USA (10.6 billion L) (ANP, 2005; Hamelinck *et al*., 2005).

Low-grade wheat flour (LG) and wheat bran (WB) are wheat milling by-products generated at the breaking rolls and size reduction system. The extraction yield of LG and WB are *c.a.* 5 % and 11 %, respectively. LG is composed of outer parts of wheat kernel, i.e. outer pericarp, aleurone layer and starchy endosperm; meanwhile the major constituents of WB are seed coat and nucellar epidermis. Basically, LG is used as supplement for animal feed. WB is used in the food industry mainly as a source of dietary fiber or metallic ions such as Ca and Mg.

Various studies about ethanol conversion systems from wheat products can be found in the literature, most of which are based on the utilization of raw wheat flour (Montesinos *et al*., 2000; Favela-Torres *et al*., 1988) or damaged wheat grains (Suresh *et al*., 1999). Unfortunately, a few reports are available on utilization of wheat milling by-products for bioethanol production (Palmarola-A. *et al*., 2005), to the best of our knowledge. Thus, the objectives of this study were (1) to develop the SSF for wheat milling by-products; (2) to evaluate the performance of LG and WB as substrate for bioethanol production by SSF; and (3) to determine whether those by-products are comparable to other feedstock commonly used for bioethanol production.

5.2 Materials and Methods

5.2.1 Raw Material

Low-grade wheat flour (LG) and wheat bran (WB) were used as substrate for fermentation, their performance was evaluated and compared to the reference substrate wheat flour (WF). The chemical composition of each substrate is presented in Table 5.1. All samples were provided by Nisshin Flour Milling Co. Ltd., Japan.

Table 5. 1 Major components of wheat products used for ethanol production (%)

Component	Wheat product		
	LG	WB	WF
Moisture	14.0 ± 0.15 [a]	12.2 ± 0.19	13.1 ± 0.01
Starch	15.6 ± 1.30	11.7 ± 2.78	62.0 ± 2.25
Ash	2.7 ± 0.01	5.6 ± 0.04	0.6 ± 0.15
Protein	15.0 [b]	13.3	10.4
Fiber	0.8	10.8	0.2
Other [c]	51.9	46.4	13.7

[a] Mean value ± Standard Deviation (n = 3)
[b] Data provided by Nisshin Flour Milling Corp.
[c] Pentosans, sugars (Klass, 1981)

5.2.2 Bacterial cells and culture media

Zymomonas mobilis NBRC 13756 commonly utilized on fermentation processes (Favela-Torres *et al*., 1988) was used as ethanol-producing bacterial strain. The bacterium was maintained in a liquid medium (pH 6.8), which composition (in g/L) was: glucose, 20; yeast extract, 5 and $MgSO_4 \cdot 7H_2O$, 2, in distilled water. Cultures were incubated at 30°C for 48 h and then stored at 4°C. This medium was also used for the pre-culture, under the same conditions.

5.2.3 Pre-cultivation

Aliquots containing 10 mL of *Z. mobilis* stock culture were used to inoculate 90 mL of the pre-culture medium in 300 mL Erlenmeyer flasks and cultivated at 30°C for 24 h, shaken at 100 rpm in aerobic condition. The cell density of starter culture was adjusted in order to obtain approximately $2x10^4$ cells/g-flour, using Optical Density for evaluation of cell density.

5.2.4 Enzymes

Commercial enzymes α-amylase and glucoamylase (as specified in Chapter 2, section 2.2.3); cellulase (EC 3.2.1.4; 106 U/mg, MP Biochemicals, USA) from *Aspergillus niger*.

5.2.5 Liquefaction

Batches of 1 L slurries containing 200 g-flour/L of each raw material, were prepared in separate; these slurries had the following pH values: LG (pH 6.1); WB (pH 5.9); WF (pH 5.7). Samples were hydrolyzed using 400 U-α-amylase/g-flour (in case of LG or WF) or 400 U-cellulase/g-flour (for WB); the liquefaction was conducted at 55°C and 100 rpm for 2 h.

5.2.6 Simultaneous Saccharification and Fermentation

After adjusting the pH of hydrolyzed slurry to 4.5, 200 U-glucoamylase/g-flour and 100 mL of *Z. mobilis* starter culture were added; the mixture was aseptically transferred to a previously sterilized 2 L-jar fermentor (MDL 200 B.E. Marubishi, Japan), which was set at 35°C, 100 rpm and pH 4.5. NaOH 3M was used for pH

control. N_2 gas (100 mL/min) was continuously bubbled to assure the anaerobic environment.

5.2.7 Analytical Methods

Glucose, maltose, ethanol and reducing sugar analysis were conducted as described previously in chapter 2 (section 2.2.4). The proximal composition of each substrate (LG, WB and WF) was analyzed using the methodology described in chapter 2 (section 2.2.7).

Viable counts were accessed by plate count method, and microbial growth rate (μ) was calculated by linear regression equations derived from each exponential growth phase (Moon, *et al*., 2005) as indicated previously (Equation 2.3).

The generation time (g) was calculated as described by Jain *et al*. (1985) (Equation 2.4).

The fermentation performance was evaluated based on ethanol production (P, g/L), productivity (Q, g/L·h), overall volumetric productivity (Q_v, g/L·h), yield ($Y_{P/S}$, g-ethanol/g-substrate), and the residue formation (R_f, g-dry solids/g-raw material), for each substrate. Ethanol production data were analyzed using an exponential law (Jain *et al*., 1985); experimental values fitted well the model with regression coefficients of more than 0.98. Q was calculated by differentiating the ethanol production data as a function of time. The overall volumetric ethanol productivity (Q_v) was calculated dividing the final ethanol production by the total fermentation time. The ethanol yield on dry basis of substrate was calculated as described previously (Equation 2.2).

After 48 h SSF the final product was centrifuged at 5,000 rpm for 30 min, the solid residue was dried and weighed in order to evaluate the residue formation (R_f).

5.3 Results and Discussion

5.3.1 Liquefaction Using Various Wheat Products

Time-course profiles of glucose, maltose and reducing sugars during the liquefaction are shown in Figure 5.1. The amount of maltose released after 1 h liquefaction of LG was comparable to that of WF despite the difference on initial starch content in each substrate (Table 5.1). The stabilization of maltose release after 2 h liquefaction of LG (Figure 5.1a) indicates that the hydrolysis time was suitable for complete starch breakdown. This is in line with the results presented by Montesinos *et al*. (2000) for purified starch liquefaction. In the other hand, in case of WF, the continuous increase on maltose concentration throughout the liquefaction (Figure 5.1c) suggests that 2 h hydrolysis were not sufficient to hydrolyze the large amount of starch present in that substrate (Table 5.1).

Despite using cellulase to break down the lignocellulosic material in WB, the hydrolysis was not complete, resulting in a slight variation in the reducing sugar content during the liquefaction. Most likely, this was due to the protection of lignin sheath and the crystalline structure of cellulose (Palmarola-A., *et al*., 2005).

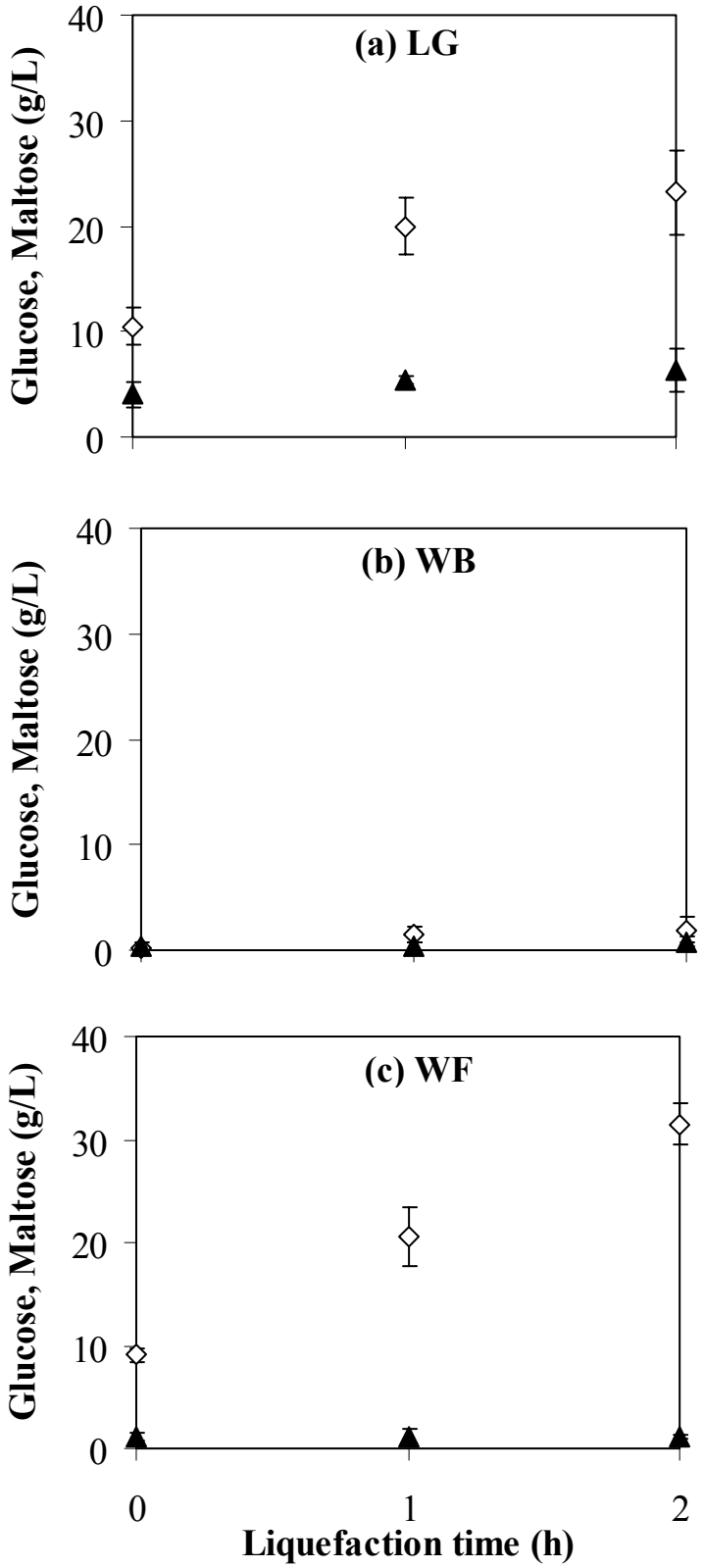


Figure 5.1 Sugar pattern during liquefaction of various wheat products. (a) LG; (b) WB; (c) WF. Symbols: ▲, Glucose; ◇, Maltose. The bars represent the standard deviation (n=3)

Furthermore, the pentosans present in wheat products are known to have low water solubility, thus reducing their availability for enzymatic hydrolysis (Palmarola-A. *et al*., 2004). In order to improve this hydrolysis efficiency different techniques have been used such as steam pressure pre-treatment combined with maceration using cellulase among others (Shiiba *et al*., 1994).

Besides releasing glucose as the major product of WB hydrolysis (Klass, *et* al., 1981), various authors mentioned the presence of arabinose and xylose (Palmarola-A. *et al*., 2005; Shiiba *et al*., 1993), two pentose sugars released from WB by cellulase. Furthermore, depending on the enzyme utilized, *e.g.* using endoglucanases, the resulting products may include cellodextrin or cellobiose, which may be further hydrolyzed to glucose in presence of β-glucosidase (Suurnäkki *et al*., 2000).

Frederiksson *et al*. (1998) reported about the gelatinization temperature (onset, peak and offset) of wheat starch as follows: 51.6 °C, 56.1 °C and 83.6 °C, respectively, determined by differential scanning calorimetry. In the present study the liquefaction was conducted at 55°C; this temperature was determined during the liquefaction optimization in our previous study (Neves *et* al., 2002, 2006). Taking into account that the liquefaction temperature used in this work falls into the gelatinization temperature range of wheat starch, it is reasonable to assume that gelatinization occurred to some extent, depending on the characteristics of each substrate utilized (LG, WB or WF); the gelatinization might play a major role during the liquefaction process due to the softening of starch granules, contributing to enzymatic hydrolysis. The high fiber content in case of WB (Table 5.1) might have caused the low liquefaction efficiency, supposing that the fibers surround the starch granules in such a fashion that they exclude water and resist enzymatic activity.

5.3.2 Simultaneous Saccharification and Fermentation of Liquefied Slurries from Various Wheat Products

The role of different wheat products on ethanol production by SSF using glucoamylase and *Z. mobilis* was investigated and the results are presented in Figure 5.2. The highest ethanol production of 68.1 g/L was obtained from WF, followed by LG and WB with 51.4 g/L and 18.1 g/L, respectively. When LG was used as substrate, glucose was promptly consumed; this might indicate the presence of easily fermentable sugars and fast carbon assimilation. The peak ethanol production from WB (18 g/L) was slightly higher than the reference value obtained from starch-free bran (13 g/L) pre-treated with 0.2% H_2SO_4 at 160°C for 20 min (Palmarola-A. *et al*., 2005). In this study, raw WB was used as substrate for liquefaction, without pre-treatment.

The high glucose concentration at the beginning of the SSF process (Figure 5.2a) was likely caused by different factors: the amount of glucose released should be higher than the initial amount of starch (Klass, *et* al., 1981), considering that a molecule of water is added across each glycosidic bond. Furthermore, the growth medium utilized for pre-culture of *Z. mobilis* contained glucose (20 g/L), which was added (10 % v/v) to the slurry before SSF.

In all cases (LG, WB and WF) the glucose production was faster than its consumption at the beginning of the fermentation (Figure 5.2). Especially in case of WB the glucose repression effect on maltose consumption could be observed, and maltose (which is not assimilated by *Z. mobilis*) might have inhibited glucoamylase activity; this inhibition was previously mentioned in the literature, during the enzymatic hydrolysis of raw wheat starch (Montesinos, *et al*., 2000).

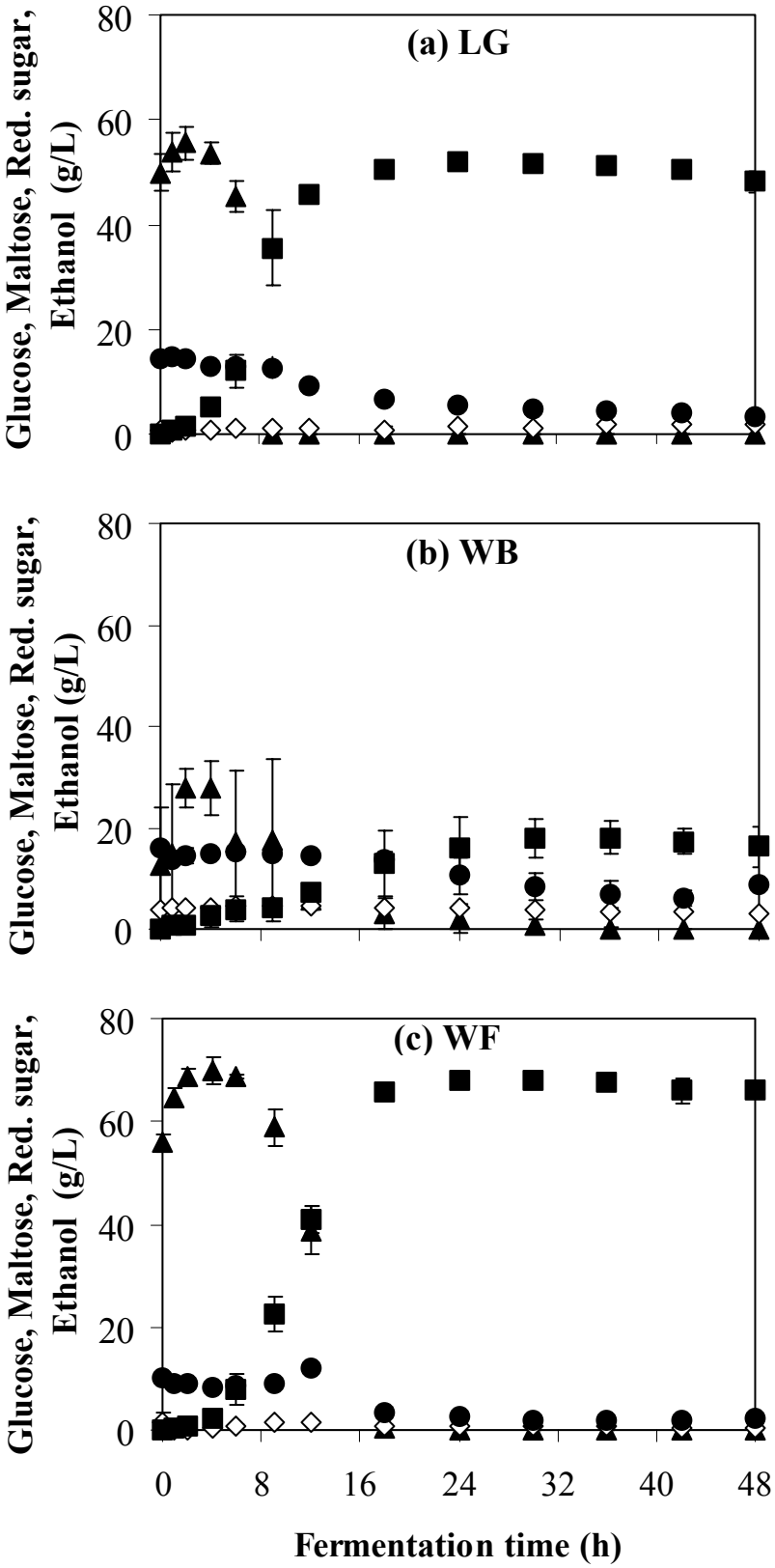


Figure 5.2 Time course of SSF from various wheat products. (a) LG; (b) WB; (c) WF. Symbols: ▲, Glucose; ◇, Maltose; ●, Reducing sugar; ■, Ethanol. The bars represent the standard deviation (n=3)

Figure 5.3 shows the time course of *Z. mobilis* growth during the SSF. When growing on a WB-based mash, after a considerable long lag phase (*c.a.* 24 h), a fast increase on cell density was observed, reaching a peak after about 36 h. The maximum cell density obtained with WB represented about 4 times that from LG. In other hand, the lag phase took a few initial hours in case of LG and WF. Initially, the sugar from WB was mostly converted into ethanol; after *c.a.* 24 h, when the exponential growth phase started, the substrate consumed was basically converted into biomass.

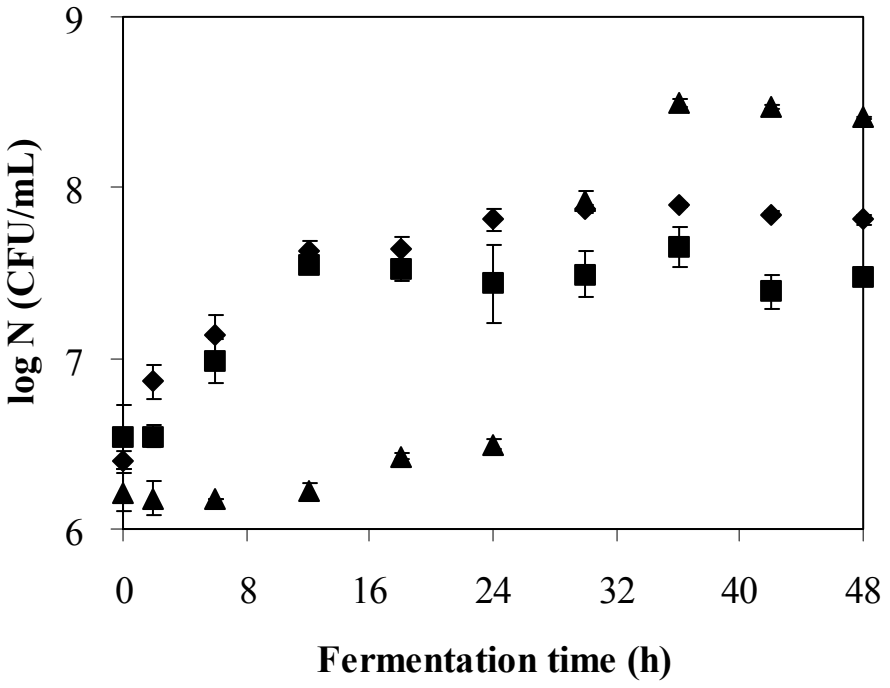


Figure 5.3 ***Z. mobilis*** **growth pattern during the SSF. Symbols: ◆, LG; ▲, WB; ■, WF. The bars represent the standard deviation (n=5)**

These results are consistent with the hypothesis that there is a coupling between *Z. mobilis* growth and ethanol production (Torres *et al.,* 1986). Along with the almost complete conversion of glucose into ethanol at WB fermentation onset, other fermentable sugars likely present in the fermentation mash (such as arabinose or xylose) were also consumed, which agrees well with the results shown in the literature

for the hydrolysis of wheat starch fibers for ethanol production (Palmarola-A. *et al*., 2004).

Furthermore, a coupling between the high fiber content in the substrate (in case of WB) and *Z. mobilis* growth was observed in the present work, considering that the delay on lignocellulosic material degradation inhibited the bacterial growth, resulting in a prolonged lag phase.

The effect of various substrates on the kinetic parameters of microbial growth is described in Table 5.2. The highest growth rate (μ) of 0.142 h^{-1} was obtained from WB, followed by LG (0.119 h^{-1}) and WF (0.043 h^{-1}). These results indicate that *Z. mobilis* grows faster when WB is used as substrate, once the fermentation time overcomes the lag phase. As for the generation time (g), also known as doubling time, *Z. mobilis* took in average 16.2 h to double its population, when growing on a WF-based fermentation mash, which was about 3.3 times longer than the generation time using WB as substrate.

Table 5.2 Kinetic parameters of microbial growth and the effect of various substrates

Kinetic parameter	Substrate		
	LG	WB	WF
Growth rate (μ) (h^{-1})	0.119	0.142	0.043
Generation time (g) (h)	5.8	4.9	16.2

5.3.3 Performance of Various Wheat Products as Substrate for Fermentation

Table 5.3 shows the results of the parameters used to evaluate the fermentation performance of each substrate (P, Q, Q_v, $Y_{P/S}$, and R_f). WF produced the highest amount of ethanol (66.6 mL), followed by LG (39.1 mL) and WB (6.5 mL). The

highest amount of solid residue of 111.2 g was generated during WB fermentation, followed by LG (28.5 g) and WF (8.3 g), respectively. WB presented a very low ethanol yield (0.024 g/g), resulting in the lowest overall ethanol productivity (1.09 g/L·h), among the three substrates tested. The ethanol yield obtained from LG (0.174 g/g-substrate) is about 6 fold that obtained from WB, and considerably higher than the theoretical ethanol yield from sugar cane (0.063 g/g) (Moreira *et al*., 1999) or cassava (0.142 g/g) (Barretts de Menezes, 1982) two major agricultural crops actually used as feedstock for fuel ethanol production, revealing the suitability of LG as feedstock for bioethanol production.

Table 5.3 Fermentation performance of various wheat products

Parameter for performance evaluation	Substrate		
	LG	WB	WF
Raw material [a]			
Substrate concentration (g/L)	200	200	200
Moisture (%)	14.0 ± 0.15[b]	12.2 ± 0.19	13.1 ± 0.01
Ash (%)	2.7 ± 0.01	5.6 ± 0.04	0.6 ± 0.15
Final product			
Ethanol production (P) (g/L) [c]	51.4 ± 0.37	18.1 ± 3.17	68.1 ± 1.43
Supernatant (mL)	600	320	760
Ethanol (mL)	39.1	6.5	66.6
Residue (R_f) (g) (dry matter)	28.5 ± 2.69	111.2 ± 8.31	8.3 ± 0.93
Ash (%)	3.9 ± 0.04	18.7 ± 0.21	7.7 ± 0.42
Ethanol yield ($Y_{P/S}$) (g/g of dry flour)	0.17 ± 0.01	0.02 ± 0.08	0.30 ± 0.03
Overall volumetric productivity (Q_v) (g/L·h)	2.72 ± 0.04	1.09 ± 0.21	3.64 ± 0.08

[a] Initially 1 L slurries were prepared from each substrate, in separate

[b] Mean value ± Standard Deviation (n = 3)

[c] Before centrifugation

The ethanol productivity of various wheat products is shown in Figure 5.4. In case of WB the ethanol productivity was lower than the two other substrates, which was likely caused by the presence of pentoses in WB (Palmarola-A. *et al*., 2005; Shiiba *et al*., 1993); *Z. mobilis* is unable to ferment such sugars, it can only ferment hexoses such as glucose, fructose or sucrose. Incomplete starch hydrolysis during WF liquefaction might have caused delay on ethanol production from WF, compared to LG. This was consistent with the hypothesis that starch hydrolysis is made unease by the increased viscosity during liquefaction, caused by starch granules swelling and water penetration (Montesinos *et al*., 2000). As for WB, besides presenting the lowest ethanol productivity among the three substrates tested (*c.a.* 1.2 g/L·h after about 12 h SSF), the final ethanol concentration (18.1 g/L) was considerably low compared to that obtained from LG (51.4 g/L) (Table 5.3).

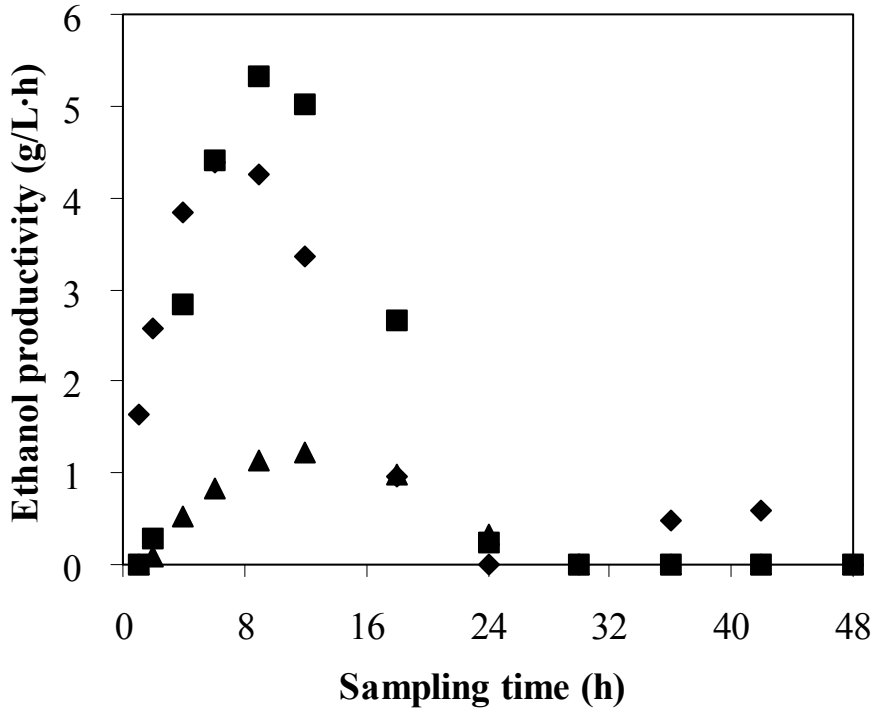


Figure 5.4 Ethanol productivity (*Q*) from various wheat products. Symbols: ◆, LG; ▲, WB; ■, WF

A summary of ethanol production from various substrates using different ethanol conversion systems and microorganisms is shown in Table. 5.4. Most of the studies were conducted in shake flasks or small volume bioreactors. The ethanol production from LG (51.4 g/L) is comparable to most studies based on wheat products (Suresh *et al*., 1999; Palmarola-A. *et al*., 2005).

LG represents about 5 % of the total WF produced during wheat milling. Nearly 6.8 million ton of WF were produced in Brazil, in the year 2000 (Moreira *et* al., 1999), resulting in about 0.34 million tons of LG. If this by-product could be fully used as feedstock for bioethanol production, the potential for producing bioethanol from LG would be 78.2 million L.

Table 5.4 Comparison of ethanol production using various substrates

Reactor (volume)	Mode	Process	Culture	Source of substrate	Substrate (g/L)	Ethanol (g/L)	Productivity (g/L·h)	Yield (g/g-flour)	Source
Jar fermentor (2 L)	Batch (21 h)	SSF	Commercial α-amylase, glucoamylase and *S. cerevisiae*	Raw wheat flour	300	67	3.19 [a]	0.454 [b]	(Montesinos *et al*., 2000)
Erlenmeyer (500 mL)	Batch (90 h)	SSF	α-amylase (from *B. subtilis*) and *S. cerevisiae*	Fine-wheat flour	250	44	0.49 [a]	0.18	(Suresh *et al*., 1999)
				Damaged wheat		34	0.38 [a]	0.14	
				Damaged sorghum		27	0.30 [a]	0.11	
Circulating loop reactor (9 L)	Fed-batch (600 h)	SSF	α-amylase (from immobilized *A. awamori*) and *S. cerevisiae*	Raw cassava starch	150	90	1.17	0.45 [b]	(Roble *et al*., 2003)
Manufactured fermentor (1.4 L)	Batch (24 h)	Single ferment.	Recombinant *S. cerevisiae*	Wheat starch pre-fermentation effluent	120 [c]	59	2.45 [a]	0.47	(Zaldivar *et al*., 2005)
Erlenmeyer (25 mL)	Batch (6 h)	Single ferment.	*S. cerevisiae*	WB	200	13	2.16 [a]	0.01	(Palmarola-A. *et al*., 2005)
Jar fermentor (2 L)	Batch (48 h)	Single ferment.	*Z. mobilis*	Jerusalem artichoke juice	250 [c]	100	1.9	0.47	(Torres *et al*., 1986)
Jar fermentor (2 L)	Batch (48 h)	SSF	Commercial α-amylase, glucoamylase, cellulase and *Z. mobilis*	LG	200	51	2.72 [a]	0.17	**This work**
				WB		18	1.09 [a]	0.02	
				WF		68	3.64 [a]	0.30	

[a] Overall ethanol productivity (g/L·h)
[b] Ethanol yield (g-ethanol/g-starch)
[c] Initial glucose concentration in the fermentation mash

Chapter 6

Reference Fermentation Using Batch or Fed-batch Mode and Dry Baker's Yeast

6.1 Introduction

This study was conducted in order to evaluate fermentation processes using batch or fed-batch mode in a small-scale bioreactor. Specific fermentation rates such as specific growth rate (μ_x), specific ethanol production rate (q_p) and specific glucose consumption rate (q_s), as well as yield parameters such as ethanol yield ($Y_{p/s}$) and biomass yield ($Y_{x/s}$) were determined.

Supposing that the starch present in the substrate was completely hydrolyzed during the preliminary enzymatic hydrolysis steps (liquefaction and saccharification), basically the source of carbon available would be glucose, which is directly converted by yeasts to form ethanol. Reference processes using glucose as substrate were accomplished aiming to evaluate the effects of substrate feeding mode (batch or fed-batch) on the fermentation.

6.2 Material and Methods

6.2.1 Raw Material

Substrates containing various levels of D(+)-Glucose (Dextrose, anhydrous, 98%; Wako, Japan) in distilled water were used for fermentation. For the batch processes, two initial glucose concentrations were utilized: 50 g/L and 100 g/L. As for the fed-batch processes, initially substrates containing 50 g/L were utilized; fresh medium containing 50 g/L was added to the reactor after 6 h fermentation.

6.2.2 Microorganisms

Dry baker's yeast *Saccharomyces cerevisiae*, commonly used in bakery and brewery industries (Saf-Instant Lesaffre, Marcq-France) was used as ethanol-producing yeast strain.

6.2.3 Fermentation

6.2.3.1 Batch Fermentation

Initially 1 L substrates containing various levels of glucose (50 g/L or 100 g/L, as described above) were prepared in distilled water, pH 4.5. The mixture was transferred to a 2 L fermentor (MDL 200 B.E. Marubishi, Japan) (previously described in section 2.2.5) and autoclaved at 121°C for 15 min. After sterilization, an inoculum containing dry baker's yeast (10 g/L) was added for fermentation. The process was conducted at 35°C for 24 h, with mild agitation (100 rpm). Nitrogen gas was bubbled into the reactor at flow rate 100 mL/min, to assure on anaerobic environment. NaOH 3M was used to maintain the pH 4.5.

6.2.3.2 Fed-batch Fermentation

Initially 1 L substrates containing 50 g-glucose/L were prepared in distilled water, pH 4.5. The mixture was transferred to a 2 L fermentor and autoclaved at 121°C for 15 min. After sterilization, an inoculum containing dry baker's yeast (10 g/L) was added and the fermentation was conducted under the same conditions used for batch fermentation (section 6.2.3.1). After 6 h fermentation, 100 mL of fresh medium previously sterilized containing 50 g/L of glucose was supplemented to the fermentation mash and the process was conducted up to 24 h.

6.2.4 Analytical Methods

Aliquots were withdrawn regularly for analysis; all samples were centrifuged at 4,000 rpm for 20 min; the supernatant was used for glucose and ethanol analysis, the remaining pellet was used for biomass estimation. Glucose and ethanol concentrations were analyzed using HPLC as described earlier (section 2.2.7). The yeast cells dry weight was determined using the standard method for determination of total solids in biomass (ASTM, 1994), as follows: after centrifugation, the supernatant was separated and 20 mL of ice-cold saline (NaCl 0.85% w/v) were added to suspend the pellet, using a vortex; the suspension was centrifuged (as described above), the separated pellet was dissolved in 1 mL of distilled water and dried to constant weight in convection oven at 105°C.

6.2.5 Specific Fermentation Rates and Production Yield

6.2.5.1 Polynomial Approximation

It was assumed that the biomass (X), ethanol (P) and glucose concentration (S) are function of time (t), as indicated bellow:

$$X = a_0 + a_1 t + a_2 t^2 + ... + a_n t^n \quad (6.1)$$

$$P = b_0 + b_1 t + b_2 t^2 + ... + b_n t^n \quad (6.2)$$

$$S = c_0 + c_1 t + c_2 t^2 + ... + c_n t^n \quad (6.3)$$

The coefficients a_0, a_1, … a_n, b_0, b_1,… b_n, c_0, c_1,… c_n were determined by least squares fitting of the experimental data. The degree n of the polynomial was chosen to get a correlation coefficient r^2>0.97; n varied between 3 (for batch processes) and 5 (for fed-batch processes).

6.2.5.2 Determination of Kinetic and Yield Parameters

Equations 6.1 to 6.3 were differentiated as function of time, obtaining:

$$\frac{dX}{dt} = a_1 + 2a_2t + ... + na_nt^{n-1} \quad (6.4)$$

$$\frac{dP}{dt} = b_1 + 2b_2t + ... + nb_nt^{n-1} \quad (6.5)$$

$$\frac{dS}{dt} = c_1 + 2c_2t + ... + nc_nt^{n-1} \quad (6.6)$$

Equations 6.4 to 6.6 were used for calculation of yield and kinetic parameters at specific time points, as described by Favela-Torres *et al*. (1986):

- Biomass yield ($Y_{x/s}$) (g-biomass/g-glucose): $Y_{\frac{x}{s}} = \frac{\frac{dX}{dt}}{\frac{dS}{dt}}$ (6.7)

- Ethanol yield ($Y_{p/s}$) (g-ethanol/g-glucose): $Y_{\frac{p}{s}} = \frac{\frac{dP}{dt}}{\frac{dS}{dt}}$ (6.8)

- Specific growth rate (μ_x) (1/h): $\mu_x = \frac{1}{X}\frac{dX}{dt}$ (6.9)

- Specific substrate consumption rate (q_s)(g-glucose/g-biomass.h) $q_s = \frac{1}{X}\frac{dS}{dt}$ (6.10)

- Specific ethanol productivity (q_p) (g-ethanol/g-biomass.h) $q_p = \frac{1}{X}\frac{dP}{dt}$ (6.11)

6.3 Results and Discussion

Substrates containing 50 g-glucose/L and 100 g/L were utilized for batch processes. For the fed-batch fermentation initially 50 g/L were used, supplemented with 50 g/L (after 6 h fermentation). The results are shown in Figure 6.1.

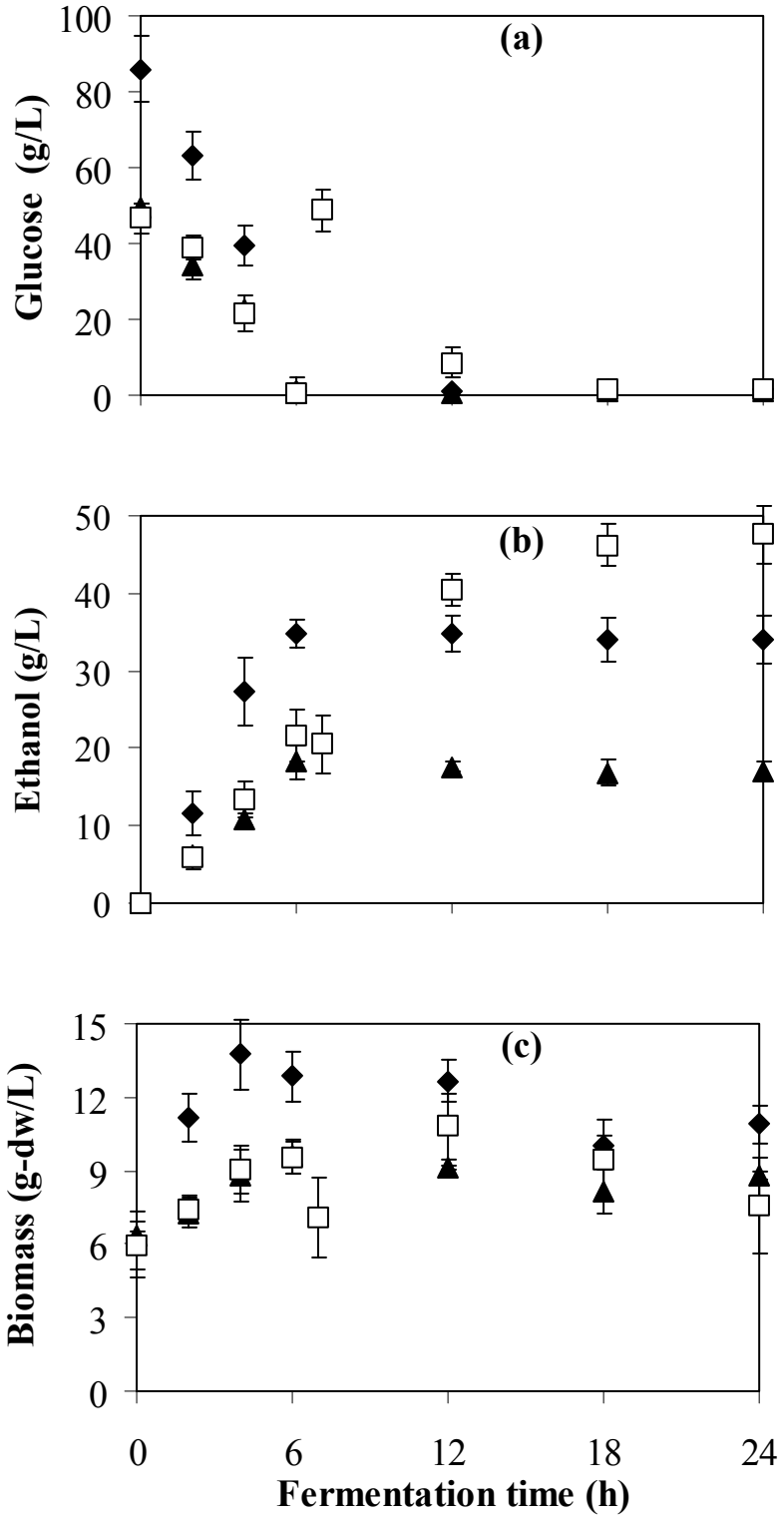


Figure 6.1 Time courses of glucose fermentation using different processes. (a) Glucose; (b) Ethanol; (c) Biomass dry weight. Symbols: ▲, Batch (50 g/L); ◆, Batch (100 g/L); □, Fed-batch (50 g/L, plus 50 g/L after 6 h). The bars represent the standard deviation (n=3)

When the fermentation was conducted using batch processes, independent of the initial glucose concentration (50 g/L or 100 g/L), after 6 h fermentation the glucose in the fermentation medium was completely consumed; hereafter there was no further ethanol production indicating the end of the fermentation, likely due to nutrient starvation. The substrate containing 50 g-glucose/L of resulted in *c.a.* 18 g-ethanol/L, representing an overall yield of 0.36 g-ethanol/g-glucose which is nearly 70 % of theoretical yield (0.51 g/g). As for the substrate containing 100 g/L of glucose, *c.a.* 35 g/L of ethanol were obtained, yielding 0.35 g-ethanol/g-glucose. In despite of a higher ethanol concentration was obtained increasing the glucose concentration in the substrate, the overall ethanol yield did not vary considerably.

No lag phase was observed in any process (batch or fed-batch), probably because baker's yeast does not require an activation period. Furthermore, higher glucose concentration in the substrate resulted in considerable increase in biomass.

Based on the fact that for batch fermentation the overall ethanol yield did not vary when the initial glucose concentration in the fermentation mesh was increased from 50 g/L to 100 g/L, fed-batch processes were conducted, aiming to increase the ethanol yield. Thus, 1 L substrates containing initially 50 g-glucose/L were used for fermentation, and 100 mL of fresh medium containing 50 g/L was supplemented after 6 h fermentation. In this case, a consistent increase in ethanol production was observed nearly throughout the process, leading to a final ethanol concentration of *c.a.* 48 g/L, which is nearly 1.3 times the ethanol production when 100 g/L of glucose was used as substrate for batch fermentation.

The experimental data were treated using least square analysis in order to evaluate the specific fermentation rates and production yield. The results of these analyses are shown in Figures 6.2 to 6.6.

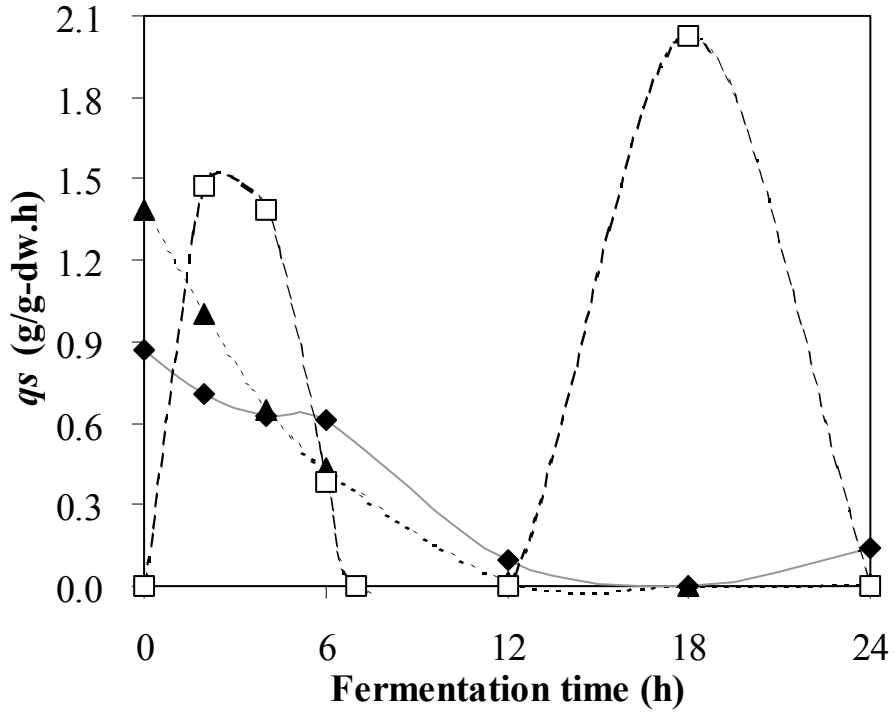


Figure 6.2 Specific glucose consumption rate (q_s) using different processes. Symbols: ▲, Batch (50 g/L); ◆, Batch (100 g/L); □, Fed-batch

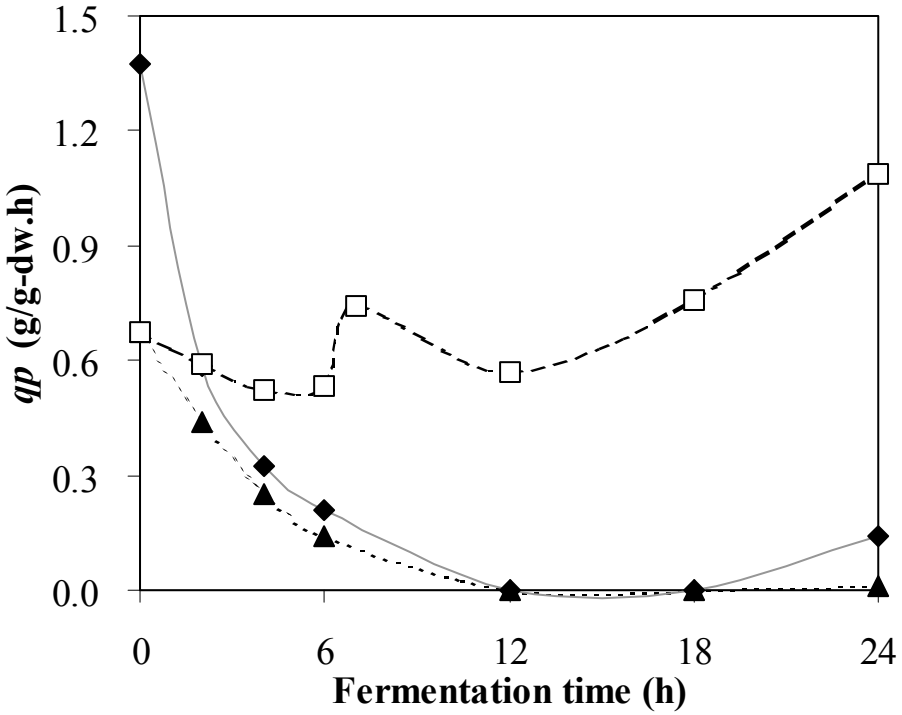


Figure 6.3 Specific ethanol production rate (q_p) using different processes. Symbols: ▲, Batch (50 g/L); ◆, Batch (100 g/L); □, Fed-batch

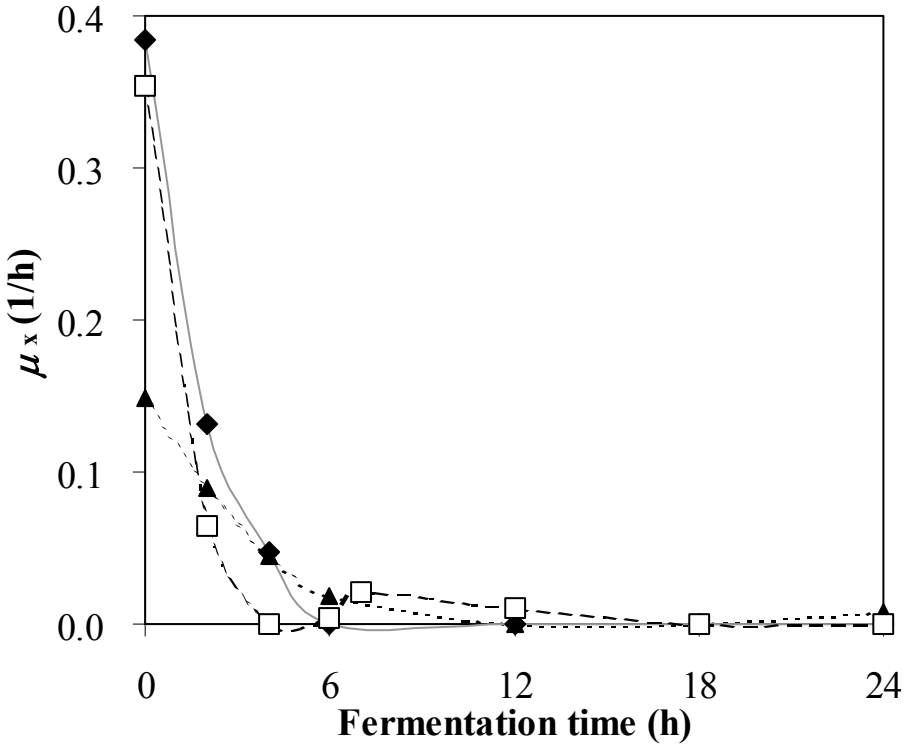


Figure 6.4 Specific growth rate (μ) using different processes. Symbols: ▲, Batch (50 g/L); ◆, Batch (100 g/L); □, Fed-batch

An attempt to characterize the ethanol production by growth associated model failed because a plot of *dP/dt vs. dX/dt* was not linear during the whole fermentation. This must be a characteristic of the ethanol-producing microorganism utilized; earlier in this manuscript (section 5.3.2) a coupling between *Z. mobilis* growth and ethanol production by SSF of low-grade wheat flour was described. In that case, the ethanol production is closely related to the bacterial growth clearly indicating that the strain initially utilized the substrate for growth and then for ethanol production. The coupling between *Z. mobilis* growth and ethanol production was reported, when Jerusalem artichoke juice containing various sugar concentrations was used as substrate for batch fermentation (Torres *et al., 1986*).

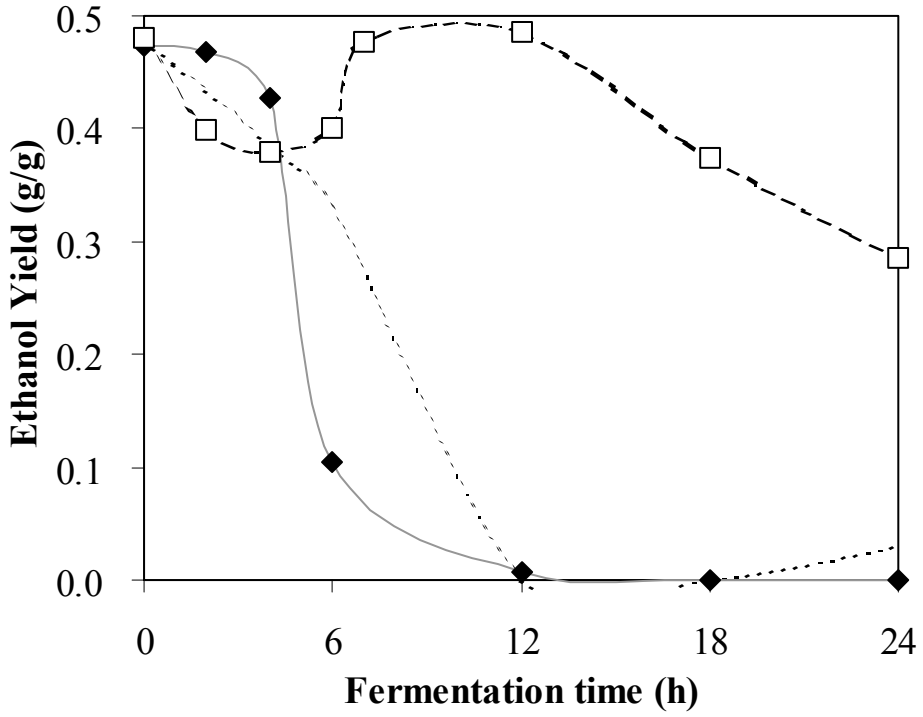


Figure 6.5 Ethanol yield using different fermentation processes. Symbols: ▲, Batch (50 g/L); ◆, Batch (100 g/L); □, Fed-batch

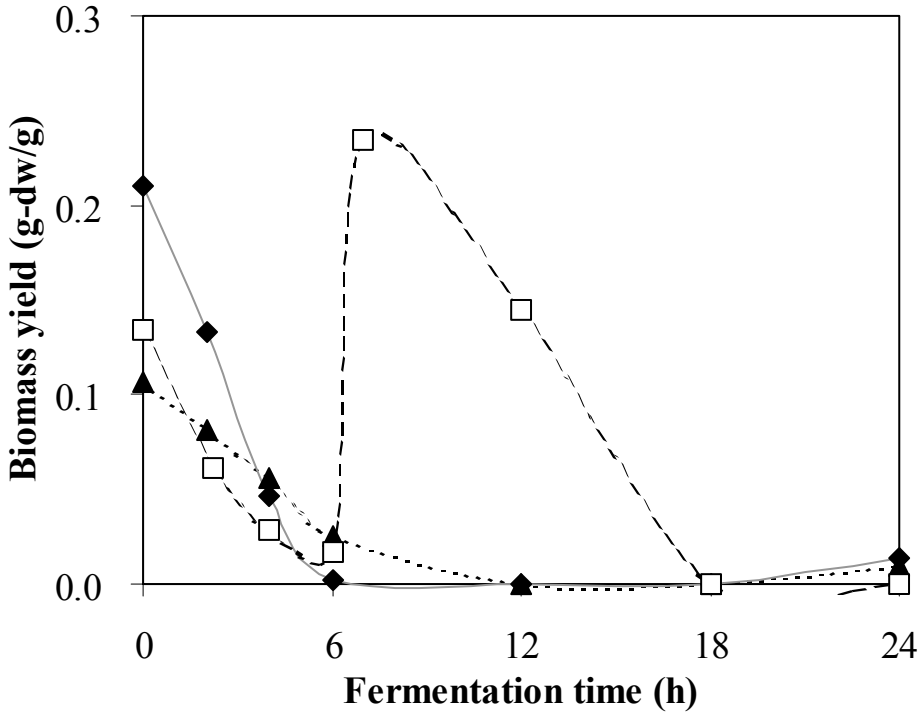


Figure 6.6 Biomass yield using different fermentation processes. Symbols: ▲, Batch (50 g/L); ◆, Batch (100 g/L); □, Fed-batch

The results above indicate that, increasing the initial glucose concentration up to 100 g/L in batch processes resulted in higher specific ethanol production rates. Moreover, increasing the glucose concentration on the substrate resulted in a delay on nutrient assimilation by the yeast, which was verified by the lower specific glucose consumption rate in case of substrates containing 100 g/L.

Considerably higher values of specific glucose consumption rate, as well as specific ethanol productivity, were obtained using fed-batch processes for fermentation, compared to batch processes

In order to compare fermentation rates between the different processes, for instance batch or fed-batch, average values had to be calculated; generally these values are obtained accounting only the experimental data obtained within the exponential growth phase. In this work we propose the use of the function biomass *vs.* time, to determine the end of the exponential phase; once the yeast reach its maximum growth, there should be no further increase in biomass, in other words, the end of the exponential phase was assumed to be the point of time when dX/dt became zero. In case of the batch processes this maximum biomass production was obtained after 4 h and 6 h for substrates containing 50 g/L and 100 g/L, respectively. As for the fed-batch process, the average parameters were calculated considering the end of exponential growth phase after 12 h fermentation. The resulting average parameters are presented in Table 6.1.

Table 6. 1 Average kinetic and yield parameters of batch and fed-batch fermentation

Parameter	Fermentation process		
	Batch (50 g/L)	Batch (100 g/L)	Fed-batch (50+50 g/L)
Time intervals for calculation (h)[a]	0-4	0-6	0-12
Specific glucose consumption rate (g/g.h)	1.02	0.74	0.73
Specific growth rate (h^{-1})	0.09	0.19	0.18
Specific ethanol production rate (g/g.h)	0.46	0.79	0.63
Biomass yield (g/g)	0.08	0.11	0.13
Ethanol yield (g/g)	0.43 ± 0.07[b]	0.30 ± 0.03	0.43 ±0.08
% of theoretical ethanol yield (%)	84.6	56.6	84.8
Final ethanol concentration (g/L)	17.1 ± 0.7	33.9 ± 1.8	47.5 ± 2.3
Overall volumetric ethanol productivity (g/L·h)[c]	3.0 ± 0.14	5.8 ± 0.42	2.0± 0.14

[a] Time intervals assumed for the exponential growth phase

[b] Mean value ± Standard Deviation (n = 3)

[c] Peak ethanol production divided by fermentation time (batch: 6 h; fed-batch: 24 h)

A suitable method for evaluation of fermentation performance using batch and fed-batch processes had to be selected. As described above, we proposed to use average fermentation rates and yield parameters, calculated during the exponential growth phase, as it should reflect the interval with maximum microbial activity. Under these conditions, the results for average specific ethanol production rate and ethanol yield obtained during the fed-batch process (0.63 g-ethanol/g-biomass·h and 0.43 g-ethanol/g-glucose, respectively) were inferior to those obtained using 100 g-glucose/L for batch fermentation (0.79 g/g·h and 0.30 g/g, respectively). Moreover, in case of fed-batch processes the specific ethanol production rate values obtained were considerably higher than those obtained by batch processes after *c.a.* 2 h fermentation.

Chapter 7

General Conclusion

The liquefaction conducted using 200 U-α-amylase/g-flour at 55°C for 2 h was found to be the most suitable process, considering the highest liquefaction yield and the amount of fermentable sugar released from low-grade flour. In the present study various levels of α-amylase and β-amylase, and several experimental conditions were tested. α-Amylase was revealed as the most suitable enzyme for the liquefaction of low-grade wheat flour.

The potential of utilizing wheat milling by-products for bioethanol production was investigated. The above results demonstrated that LG and WB, two by-products of wheat milling process, were used successfully as substrate for fermentation. It was concluded that LG has the potential to serve as a low cost feedstock for bioethanol production, with a fermentation performance comparable to that of WF and an ethanol yield superior to other agricultural crops.

Increasing the substrate concentration up to 200 g/L resulted in improved performance during fermentation, considering as parameters the ethanol yield, productivity and the microbial growth.

Regarding the performance of WB (the major by-product generated during wheat milling), the process utilized was not efficient to break down the cellulose into fermentable sugars, resulting in low ethanol yield (0.30 g-ethanol/g-flour). To recover the maximum amount of sugars from WB, different pre-treatment conditions are required. In order to get better fermentation performance from this substrate, the use of hemicellulolytic enzymes

associated with increased hydrolysis time and temperature might be required. Hence, the detailed mechanism of the enzymatic hydrolysis of WB lignocellulosic material is yet to be fully understood.

A process involving the simultaneous saccharification and fermentation of wheat milling by-products was developed. Based on the high fermentation rate of LG, reaching the peak ethanol productivity of 4.3 g/L.h after 9 h of SSF, a considerable profit on fermentation time was achieved, compared to industrial processes, which usually take at least 80 h to complete (Aiba, 1983). When fine wheat flour was used for fermentation (Montesinos *et al*., 2000) the peak ethanol productivity was 3 g/L.h, which is about 10 % higher than LG. The maximum ethanol production of 51.4 g/L obtained from LG is about 50 % higher than that produced from damaged wheat flour (Suresh *et al*., 2000). The ethanol yield from LG (0.18 g/g-flour) is relatively higher than the yield from sugar cane or cassava (Moreira *et* al., 1999; Barretts de Menezes, 1982).

The process proposed is an efficient method to achieve direct ethanol production from wheat milling by-products, reaching levels of ethanol productivity and yield comparable to various agricultural products in a short-time process, resulting in reduced net energy consumption. By adjusting the initial liquefaction process this technology can be applied to other starch crops and crop residues, potential feedstock for bioethanol production.

As for now, under the experimental conditions utilized, i.e. specific enzyme, microorganism, hydrolysis time, temperature, agitation and small volume reactor, we were able to produce up to 0.18 g-ethanol/g of low-grade wheat flour, with an overall volumetric ethanol productivity of 2.72 g/L·h.

The production of ethanol by fermentation of glucose obtained via enzymatic hydrolysis of wheat milling by-products considered in this work requires further research and development before economic feasibility can be attained. In special, additional effort should done in order to optimize α-amylase production in the laboratory, as attempt to lower the overall cost of the process, considering that the use of commercial enzymes for fuel ethanol in large scale is not economically viable. The use of immobilized cells for continuous enzyme production should be a suitable alternative for reaching higher productivities.

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Bioethanol Production from Wheat Milling By-products

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Summary

The state of the art of starch hydrolysis and fermentation technologies to produce ethanol from wheat by-products was evaluated. Two samples of low-grade wheat flour, namely low-grade 1 (LG1) and low-grade 2 (LG2), with different carbohydrate and fibrous content, were used as substrates. The samples were liquefied using various concentrations of α- or β-amylase, in order to optimize the production of fermentable sugars; the enzyme α-amylase revealed higher performance. After liquefaction, glucoamylase was used for saccharification, and dried baker's yeast for fermentation, simultaneously (SSF). Glucose was consumed promptly in both cases, LG1 and LG2; meanwhile the ethanol production was considerably higher in LG1 (38.6 g/L), compared to LG2 (24.9 g/L). The substrate with LG1 revealed higher potential as substrate for ethanol production.

Low-grade wheat flour (LG) was used as substrate for fermentation, at three different levels: 100, 200 or 300 g-flour/L, in distillate water. The samples were liquefied using α- amylase (400 U/g-flour) at 55°C for 2 h. The SSF was then conducted in a jar fermentor, using glucoamylase for saccharification, and *Z. mobilis* NBRC 13756 for fermentation, at pH 4.5, 35°C for 48 h. Cell density, along with other kinetic parameters such as the microbial growth rate (μ), the ethanol yield ($Y_{P/S}$) and productivity (Q_v) were also evaluated. When substrates containing 200 g/L were utilized, a peak ethanol production (P) of 51.5 g/L was obtained after 24 h fermentation, with an average yield of 0.26 g-ethanol/g-substrate.

The performance of two wheat milling by-products, low-grade wheat flour (LG) and

wheat bran (WB), as substrates for fermentation was evaluated and compared to wheat flour (WF), used as reference. Slurries containing 200 g/L of each substrate were prepared separately and hydrolyzed using α-amylase (for LG and WF) or cellulase (for WB). After liquefaction, the enzyme glucoamylase was added for saccharification and *Z. mobilis* NBRC 13756 was used as ethanol-producing bacteria. In order to evaluate the fermentation performance of each wheat product, the ethanol production (P, g/L), ethanol productivity (Q, g/L·h), overall volumetric ethanol productivity (Q_v, g/L·h), ethanol yield ($Y_{P/S}$, g-ethanol/g-substrate), and the residue formation (R_f, g-dry solids) were considered. After 24 h of SSF, the maximum ethanol production from LG was 51.4 g/L, which is approximately 2.5 fold that obtained from WB. The overall volumetric ethanol productivity from LG was 2.72 g/L.h, and the ethanol yield was 0.17 g/g-substrate, which is about 8 fold the yield from WB, and relatively higher than other agricultural crops used as feedstock for fuel ethanol production, such as sugar cane (0.06 g/g) or cassava (0.14 g/g).

Kinetics parameters of batch or fed-batch fermentation processes in a small-scale bioreactor were evaluated. Specific fermentation rates, such as specific growth rate (μ), specific ethanol production rate (*qp*) and specific glucose consumption rate (*qs*), as well as yield parameters, such as ethanol yield ($Y_{p/s}$) and biomass yield ($Y_{x/s}$) were determined. Reference processes were conducted, using substrate with different glucose concentrations, in order to evaluate the effects of the substrate feeding mode (batch or fed-batch) on the fermentation efficiency. As for processes conducted in batch mode, the substrate containing 50 g/L resulted in *c.a.* 18 g /L of ethanol, with an yield of 0.36 g-ethanol/g-glucose which is about 70 % of theoretical yield. As for the substrate containing 100 g/L, *c.a.* 35 g/L of ethanol were obtained, yielding 0.35 g/g. In case of fed-batch processes, the final ethanol concentration was *c.a.* 48 g/L, which is nearly 1.3 times the ethanol production when 100 g/L was used. Considerably higher values of specific glucose consumption rate, as well as specific ethanol productivity, were obtained by fed-batch, compared to batch fermentation.

小麦製粉副産物からのエタノール生産

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要旨

発酵のための基質として小麦製粉副産物の基質濃度を最適化することによって潜在的なエタノール生産利用のありかたを示すことが出来ると考えられる. 小麦製粉副産物の二つの異なったサンプル末粉 1（LG1）または末粉 2（LG2）を基質として用いた．澱粉の分解を最適化するためα-アミラーゼ、またはβ-アミラーゼを基質の 1 g 当たり、200 U、400 U、800 U の三つの濃度を使用し、液化を行った．α-アミラーゼはより高い性能を持つことと､最適酵素濃度は 800 U/g であることが明らかにされた. 次いでアミログルコシダーゼと乾燥パン酵母を使用し、同時糖化発酵（SSF）を行った．SSF の少時間に酵母が両方のサンプル（LG1 または LG2）に含まれてるグルコースを消費し､SSF の 24 時間後に気質として LG1 を使った場合のエタノール生成量(38. 6 g/L) が LG2 の場合 (24.9 g/L) より高かった. これらの結果は末粉の成分すなわち、LG1 の方は糖質が高く、LG2 の方は繊維含量が多いためと考えられる.

炭素源としてグルコースを使用し、乾燥パン酵母によってモデル発酵を行った．基質濃度によらず、全体のエタノールの収率（$Y_{P/S}$）はほぼ安定していた．基質としてグルコースの 50 g/L を使用した場合、$Y_{P/S}$ が 0.36 g-ethanol/g-glucose、グルコースの 100 g/L の場合、$Y_{P/S}$ が 0.35 g/g であった．基質としてグルコース 100 g/L を使用した場合、エタノールの生産性（Q_v）は 3.48 g/L･h に達した．LG を基質として 3 段階の濃度（100 g/L、200 g/L、300 g/L）のスラリーを用いて *Zymomonas mobilis* によって同時糖化発酵（SSF）を行った．小麦粉（WF）を参照基質として使用し、エタノール濃度（P)、Q_v 、$Y_{P/S}$、エタノールの生産率（Q_p)、グルコース消費率（Q_s）および

残渣（R_f）に基づいて、LG と WF の発酵性能を評価した．SSF の 12 時間後、基質濃度 100 g/L の場合、P が約 33 g/L Q_v が 2.75 g/L･h で、これらの値はグルコースの 100 g/L を使用した場合と同等であった．

ブラジルでの末粉の発生量が約 0.34 百万トン（2000 年）であり、原料として全量使用できると仮定すると 78.2 百万リットルのエタノールが生産されるものを見積られる．