THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Ethanol Production from Cellulosic Biomass by Encapsulated *Saccharomyces cerevisiae*

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Cover image: A picture of encapsulated yeast *S. cerevisiae*

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ABSTRACT

Unstable oil markets with rising environmental concerns have revived widespread interest in production of fuel ethanol from renewable materials. Cellulosic materials are abundant and prominent feedstocks for cheap ethanol production. However, due to recalcitrant structure of these materials, pretreatment is a prerequisite. Depending on the biomass, pretreatment and hydrolysis conditions, a number of degradation products and/or toxic components may be released that show strong inhibitory effects on the fermenting microorganisms. This thesis deals with application of encapsulation technology to ferment the highly toxic hydrolyzates without further pretreatment.

Free cells could not tolerate presence of 5 g/l furfural in defined medium, and inhibitors in wood and peel hydrolyzates in batch mode of operation and fermentation failed. Continuous cultivation of wood hydrolyzate was only successful at 0.1 h^{-1} and the majority of cells lost their viability after 5 retention times.

Encapsulated cell system could successfully ferment the synthetic medium containing 5 g/l furfural during sequential batch cultivations with ethanol yield of 0.41-0.42 g/g. Cultivation of undetoxified hydrolyzates was also carried out, where glucose and mannose were converted within 10 h without significant lag phase. However, a gradual decrease in cell activity was observed in sequential batches. Continuous cultivation was more successful, and wood hydrolyzate was fermented to ethanol by encapsulated *S. cerevisiae* at dilution rates up to 0.5 h^{-1}. More than 75% of the encapsulated cells were viable in the worst conditions. Ethanol was produced with yield 0.44 g/g and specific productivity 0.14–0.17 g/g·h at all dilution rates.

Contrary to wood hydrolyzate, where there is no preference for permeation of sugars or inhibitors through the capsules' membrane, encapsulation technology was applied to eliminate inhibition of limonene in fermentation of orange wastes to ethanol. The capsules' membrane, of hydrophilic nature, is practically impermeable to hydrophobic compounds such as limonene while allowing penetration of nutrients and products. While presence of 0.1% v/v limonene in the medium results in strong inhibition or even failure of cultivation with free cells, using this technique allowed fermentation of a medium containing 1.5% v/v limonene.

The impact of encapsulation on the anaerobic growth pattern, morphological and physiological changes of *S. cerevisiae* over long-term application was investigated. The growth rate, total RNA and protein content of the encapsulated cells decreased gradually over repeated batch cultivations, while stored carbohydrates content increased. Within 20 batch cultivations, total RNA and protein content of encapsulated cells decreased by 39% and 24%, whereas glycogen and trehalose content increased by factors of 4.5 and 4, respectively.

Keywords: encapsulated cells, ethanol, *S. cerevisiae,* dilute-acid hydrolyzate, *in situ* detoxification, furfural, orange peel, limonene, experimental design, immobilization

LIST OF PAPERS

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text.

I. **Talebnia, F.,** Pourbafrani, M., Lundin, M., Taherzadeh, M.J., (2008). Optimization Study of Citrus Waste Saccharification by Dilute-acid Hydrolysis, **BioResources,** vol. **3**(1), 108-122.

II. **Talebnia, F.**, Niklasson, C., Taherzadeh, M.J., (2005). Ethanol production from glucose and dilute-acid hydrolyzates by encapsulated *S. cerevisiae,* **Biotechnology and Bioengineering,** vol. **90**, No.3, 345-353.

III. **Talebnia, F.**, Taherzadeh, M.J., (2006). In situ detoxification and continuous cultivation of dilute-acid hydrolyzate to ethanol by encapsulated *S. cerevisiae*, **Journal of Biotechnology,** vol. **125**, 377-384.

IV. **Talebnia, F.**, Taherzadeh, M.J., (2007). Physiological and Morphological Study of Encapsulated *S. cerevisiae*, **Enzyme and Microbial Technology**, vol. **41**, 683-688.

V. Pourbafrani, M., **Talebnia, F.**, Niklasson, C., Taherzadeh, M.J., (2007). Protective Effect of Encapsulation in Fermentation of Limonene-contained Media and Orange Peel Hydrolyzate, **International Journal of Molecular Science,** vol. **8**, 777-787.

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"The universe is full of magical things,

patiently waiting for our wits

to grow sharper."

Eden Phillpotts

1. INTRODUCTION

At the beginning of the 20th century, many industrial materials such as dyes, solvents, and synthetic fibers were made from trees and agricultural crops. By the late 1960s, however, many of these bio-based chemical products were replaced by petroleum derivatives which could be produced at lower cost (Ragauskas et al., 2006; van Wyk, 2001).

Over the last few decades, the negative impacts of fossil fuel on the environment and consequent global warming, progressive demand for energy, inevitable depletion of the world's energy supply, and the unstable oil market (such as the energy crisis of the 1970s) have renewed the interest of society in searching for alternative fuels (Himmel et al., 2007; Solomon et al., 2007). The alternative fuels are expected to satisfy several requirements including substantial reduction of greenhouse gas emission, worldwide availability of raw materials, and capability of being produced from renewable feedstocks (Hahn-Hägerdal et al., 2006). Production of fuel ethanol from biomass seems to be an interesting alternative to traditional fossil fuel, which can be utilized as a sole fuel in cars with dedicated engines or in fuel blends. Ethanol is currently produced from sugars, starches and cellulosic materials. The first two groups of raw materials are currently the main resources for ethanol production, but concomitant growth in demand for human feed similar to energy could make them potentially less competitive and perhaps expensive feedstocks in the near future, leaving the cellulosic materials as the only potential feedstock for production of ethanol (Taherzadeh and Karimi, 2007). Cellulosic materials obtained from wood and agricultural residuals, municipal solid wastes and energy crops represent the most abundant global source of biomass (Lin and Tanaka, 2006). These facts have motivated extensive research toward making an efficient conversion of lignocelluloses into sugar monomers for further fermentation to ethanol.

Unlike simple sugars which can be directly converted to ethanol, other biomasses such as starches, lignocelluloses and citrus waste need to be pretreated to make sugars available for the subsequent fermentation step. Pretreatment and hydrolysis release a mixture of fermentable sugars as well as potential inhibitory compounds present in the structure of biomass – for instance, phenolic constituents from extractive in wood and limonene from orange peel. Hydrolysis using appropriate enzymes represents the most effective method to liberate simple sugars from cellulosic materials; however, improvement of process rates and lower cost of enzyme production are required. Dilute-acid hydrolysis is a fast and economically feasible approach that is widely used. However, dilute-acid hydrolysis is carried out at high temperature and pressure and causes formation of many by-products, depending on the source of biomass due to sugar degradation. These by-products have sometimes severe inhibitory effects on microorganisms such as *S. cerevisiae* in the subsequent fermentation stage.

1.1. Scope

High cell concentration in toxic media such as dilute-acid lignocellulosic hydrolyzate can increase the tolerance of cells against the inhibitors. Immobilizing yeast cells can provide high cell density in the bioreactor. Among the different methods of immobilization, cell encapsulation might be a promising method that has advantages over conventional immobilizing techniques (Chang et al., 1996; Park and Chang, 2000). The cell concentration in the capsules can be much higher than in gel-core beads due to better availability of space (Cheong et al., 1993; Yoo et al., 1996). A literature survey has revealed that very few reliable data are available on the use of encapsulated cells for ethanol production from toxic media such as wood or orange peel hydrolyzates. The current work is dedicated to developing a strategy to overcome the inhibitory effects of hydrolyzates for efficient fermentation of ethanol. Application of encapsulated yeast cell technology for direct fermentation of highly toxic media such as wood and orange peel hydrolyzates at high ethanol productivity was investigated. Several aspects of encapsulated yeast cells such as growth pattern, physiological and morphological changes as well as the impact of membrane and its selectivity for inhibitors were characterized in this work.

1.2. Outline

The summary of this thesis consists of seven chapters. It covers characterization and application of encapsulated yeast cells for ethanol production from synthetic defined medium, dilute-acid hydrolyzate as well as enzymatic orange peel hydrolyzate. Chapter 1 describes the introduction and motivation of this research. Chapter 2 gives a background about ethanol as a fuel and reviews the current market of ethanol in the world as well as environmental benefits of ethanol production from renewable biomasses. Chapter 3 provides an overview of the ethanol process where several steps of ethanol production from raw materials are described, and dilute-acid hydrolysis of orange peel and process optimization with the aid of a statistical approach is illustrated. The methodology that has been used through the entire work is given in Chapter 4. Chapter 5 focuses on the fermentation of undetoxified hydrolyzates using free and encapsulated yeast cells in both batch and continuous mode of operations. Application of capsules' membrane as a selective barrier for elimination of limonene toxicity in fermentation medium is also covered in this chapter. Chapter 6 deals with the physiological and morphological study of encapsulated cells, through which more details on growth pattern and responses of yeast cells to the altered environmental condition are provided. Finally, conclusions and suggestions for future studies are presented in Chapters 7 and 8, respectively.

2. ETHANOL FROM CELLULOSIC MATERIALS

2.1. Bioethanol and its application as a fuel

Ethanol, also known as ethyl alcohol with the chemical formula C_2H_5OH, is a flammable, clear, colorless and slightly toxic chemical compound with acceptable odour. It can be produced either from petrochemical feedstocks by the acid-catalyzed hydration of ethene, or from biomass feedstocks through fermentation. On a global scale, synthetic ethanol accounts for about 3-4% of total production while the rest is produced from fermentation of biomass – mainly sugar crops, e.g. cane and beet, and of grains (mainly corn) (Licht, 2006).

Ethanol as a neat fuel or even in the blended form with gasoline has a long history as automotive fuel. In 1860, German inventor Nicholas Otto used ethanol as a fuel in an early prototype of an internal combustion engine because it was widely available throughout Europe for use in spirit lamps. A few years later, Henry Ford built his first automobile with an engine that could run on ethanol. In 1908, Ford unveiled his Model T engine equipped with carburetors that could be adjusted to use alcohol, gasoline or a mixture of both fuels (Solomon et al., 2007). Ethyl alcohol as "the fuel of the future" was presented by him for the first time. In 1925, he told the *New York Times*: “The fuel of the future is going to come from fruit like that sumac out by the road, or from apples, weeds, sawdust – almost anything… There is fuel in every bit of vegetable matter that can be fermented. There's enough alcohol in one year's yield of an acre of potatoes to drive the machinery necessary to cultivate the fields for a hundred years.” However, fossil fuels were predominantly used for automobile transportation throughout the last century, obviously due to their lower production cost[*]. As an automotive fuel, hydrous ethanol can be used as a substitute for gasoline in dedicated engines. Anhydrous ethanol, on the other hand, is an effective octane booster when mixed in blends of 5 to 30% with no engine modification requirement (Licht, 2006).

2.2. World market

Today, bioethanol is the most dominant biofuel and its global production showed an upward trend over the last 25 years with a sharp increase from 2000. As of 2005, worldwide production capacity for bioethanol fuel was about 45 billion liters per year, with approximately 15% annual growth between 2000 and 2005 (Fig. 2.1). This value increased to 49 billion liters in 2006, when the Americas produced 75% of the total world

[*] Summarized from http://www.hempcar.org/ford.shtml

ethanol output, followed by Asia/Pacific and Europe/Africa with respective values of 15 and 10% (Licht, 2006). In the same period, development in two other major sectors of the ethanol market, i.e. industry and beverages, was less dynamic. The industrial alcohol market showed a rather modest rate of growth similar to the increase in Gross Domestic Product in many countries. The market for beverage alcohol in most developed countries is stagnating or even declining, due to increased health awareness*.

Fuel ethanol production is predicted to have the strongest increase in the Americas, where the production is expected to rise to around 75 billion liters by 2015, representing about 42 billion liters increase in the projection period. In Asia this value is anticipated to increase to 8 billion liters during the same period, and in Europe, with the policy of increasing the share of biofuels in the transportation sector, the production will rise strongly. Therefore, total output in 2015 is forecast to reach over 115 billion liters (Licht, 2006).

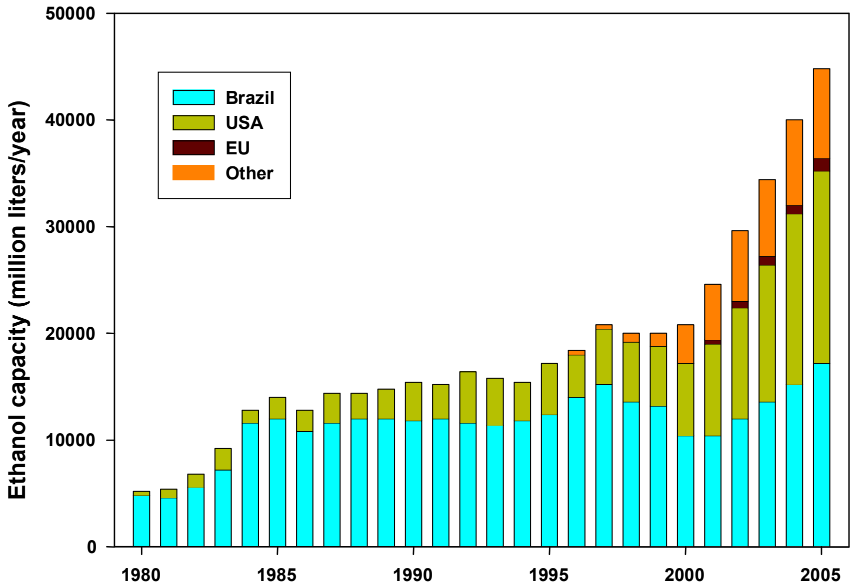

Fig. 2.1. Annual ethanol production by the major producers, adapted from Mabee (2007).

Brazil as one of the main producers in the world produces ethanol mainly from sugarcane. Because of Brazil's optimal climate, two seasons of sugarcane growth can be achieved, providing a great potential of producing both sugar and bioethanol at lower prices. Originally, the Brazilian alcohol industry was an offshoot of the sugar industry, but after world oil prices soared in 1974, the country started the world's first major program (so-called Proalcool) for the production of renewable fuels in 1975*. In 2005, Brazil produced a total of 16.82 billion liters of ethanol. Brazilian ethanol is most likely

* Summarized from http://www.distill.com/berg/

the cheapest in the world, with an estimated production cost in the range of $ 0.19-0.21 per liter in 2005 (Otero et al., 2007). The huge availability of raw material, which is sugarcane with 423 million metric tons production in 2005-06, has made Brazil the most cost-effective ethanol producer in the world[*]. Brazil controls more than 75% of the world's export market, with primary exports going to the USA, Europe, Korea, and Japan, and total exports estimated to reach approximately 3.1 billion liters in 2006 (Licht, 2006; Mabee, 2007). Brazilian gasoline has a legal alcohol content requirement ranging from 20-25% according to Renewable Fuel Standards. Most vehicles are being run on E20 or E22, but sales of flex-fuel vehicles capable of operating on E85 blends are strong (Mabee, 2007).

As shown in Fig. 2.1, bioethanol manufacturing in the *USA* was initiated in the 1980s and its fuel ethanol market is the fastest-growing market in the world. The main raw material for the US bioethanol industry is corn and to a lesser extent wheat. Most bioethanol production capacity is located in the Midwest, where corn is found in abundance. The recent policy developments in the USA stem from the Energy Policy Act of 2005, H.R. 6, which created a nationwide renewable fuels standard (RFS) that will increase the use of biofuels to 28.4 billion liters /year by 2012 (Mabee, 2007).

The largest producer of ethanol in Asia is *China* with a total alcohol production of approximately 3.65 and 3.80 billion liters in 2004 and 2005, respectively. The corresponding share of fuel ethanol was 1.25 and 1.30 billion liters (Licht, 2006). The world's largest ethanol plant, "*Jilin Tianhe*" with an initial capacity of 600,000 tons/year (2.5 million liters/day) is located in China. Ethanol has been under development in China for some time to be used as a vehicle fuel. China is planning to promote ethanol-based fuel and to create a new market for its surplus grain with the aim of reduction of oil consumption[†].

India is the second largest producer of ethanol in Asia and one of the biggest sugar producers in the world. Estimated alcohol production in 2004 and 2005 was 1.65 and 1.70 billion liters of which 100 and 200 million liters were fuel ethanol, respectively (Licht, 2006). India's transport sector is growing rapidly, demanding over half of the oil consumption in the country. Large quantities of molasses from the sugar industry motivated the government to embrace a bioethanol program for several years. Utilization of E5 blend became mandatory in 9 states and territories in the beginning of 2003[†].

2.2.1. European Union (EU)

Currently there are three major fuel ethanol producers in the EU, namely France, Spain and Sweden. While France was a dominant producer for a long time, production in Spain

[*] Adapted from http://www.marketresearch.com/map/cat/1653_2.html
[†] Adapted from http://www.distill.com/World-Fuel-Ethanol-A&O-2004.html

was sharply increased after 2000 and at the moment Spain is the largest producer in the EU. In Sweden, production of fuel ethanol started around 1920 and peaked in 1950 with an overall output of 53 million liters per year. The main feedstock was waste sulphite liquor, which is a by-product of paper manufacturing industry (Licht, 2006). Agroethanol, located in Norrköping, is the largest plant that produces ethanol from grains, with an annual capacity of 55 million liters*, while the first cellulose-based ethanol pilot plant was inaugurated in 2004 at Örnsköldsvik with a capacity of 400-500 liters/day. Sweden has more than 20 years of experience with ethanol for use in public transportation, such that today there are over 600 ethanol-operated buses in service in Sweden. At present there are over 1000 E85 pumps throughout the country, with intention to increase to more than 2,400 by 2009†. The total number of fuel-flexible cars in Sweden as estimated in 2007 exceeds 100,000§.

2.3. Environmental impacts

As long as global demand for energy continues to grow, oil prices are not likely to decrease. Consequently, the demand for a renewable and environmentally benign fuel will increase. Over the last 150 years, human activities have caused a dramatic increase in the emission of a number of greenhouse gases such as CO_2, which has led to changes in the equilibrium of the earth's atmosphere (Galbe and Zacchi, 2002). Fuel ethanol is suggested as a sustainable fuel which can be produced from renewable resources and led to maintain or even reduce the level of greenhouse gases. The net emissions of CO_2 are reported to be close to zero, since the CO_2 released during ethanol production and combustion is recaptured as a nutrient by the crops and plants which are the raw materials for ethanol production. Ethanol in blend with gasoline increases octane and provides oxygen to promote more complete combustion. Addition of ethanol or derivative such as methyl tertiary butyl ether (MTBE) to gasoline as an oxygenate reduces tailpipe emissions of CO and unburned hydrocarbons, which can contribute to improving the urban air quality. Unlike MTBE, which is not readily biodegradable and is known as a groundwater pollutant, ethanol is a water-soluble and biodegradable compound and therefore is relatively harmless to the environment, ground water and soil (Isenberg, 1999; Wyman, 1999). However, because in addition to solar energy, other energy inputs (often in the form of fossil fuel) are required in the manufacturing and marketing of biofuel such as ethanol, the entire process is not likely to be completely carbon-neutral (Granda Cesar et al., 2007).

A large number of assessments performed to estimate the environmental merit of biofuels show contradictory results (Niven, 2005; von Blottnitz and Curran, 2007). The reason may be that such assessments are generally related to the net energy value (defined as difference in the energy content of ethanol and co-products with the fossil fuel inputs)

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*Data available at http://www.agroetanol.se/aetanol.nsf/masterE?OpenFrameSet
†Data retrieved from http://www.sekab.com/default.asp?id=1844&refid=1958&l3=1949
§Data retrieved from http://di.se/Avdelningar/Artikel.aspx?stat=0&ArticleID=2008/02/25/272116

and to ethanol production routes including the type of raw materials and technologies applied. The "greenness" of a biofuel like ethanol is therefore highly dependent upon the efficiency of all stages in the process from raw material to the end use of product and its avoided use of fossil fuels. While corn ethanol is claimed to have either negative energy content or slightly positive value, environmental benefits of cellulosic ethanol cannot be refuted as the corresponding estimated net energy is rather high (Farrell et al., 2006; Granda Cesar et al., 2007; Kim and Dale, 2005).

In order to get an overall picture of the environmental impact of biofuel, emissions other than CO_2 including nitrogen oxides (NO_x), carbon monoxide (CO), sulfur oxides (SO_x), total particulate matter (TPM), and volatile organic compounds (VOCs), which could be hydrocarbon emissions, as well as other VOCs such as aldehydes, alcohols, ethers, esters, and other organics, need to be considered as well. Although ethanol (used as E85) generally generates less emissions in tailpipe (Wu et al., 2004), life-cycle analysis covering entire routes from crop to wheel may give opposite results. Even though the greenhouse gas emissions are lowered when ethanol from different routes is used and compared to gasoline, it may result in high increases of TPM, NO_x and SO_x emissions. Cellulose-based ethanol substantially decreases greenhouse gas emissions and most other pollutants, but VOCs and NO_x emissions may increase by substitution of gasoline with cellulosic ethanol. Nevertheless, cellulosic ethanol seems to be a promising choice from the perspectives of both net energy gain and overall emissions of contaminants (Granda Cesar et al., 2007; von Blottnitz and Curran, 2007).

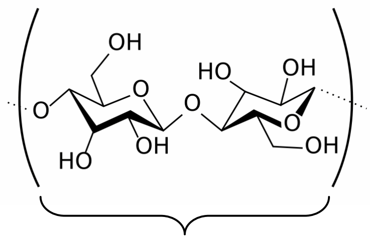
2.4. Raw materials for ethanol production

2.4.1. Lignocelluloses

Lignocellulosic materials such as agricultural and forest residues, crops and herbaceous materials in large quantities are available in many countries with various climatic conditions, making them suitable and potentially cheap feedstocks for sustainable production of fuel ethanol. The global production of plant biomass, with over 90% lignocellulose content, is estimated to be about 200×10^9 tons/year, where about $8\text{-}20\times10^9$ tons of primary biomass remain potentially accessible annually (Lin and Tanaka, 2006). Over the last few decades, extensive attention has been devoted to research on the conversion of lignocellulosic materials to ethanol (Chandrakant and Bisaria, 1998; Prasad et al., 2007). Lignocelluloses are complex mixtures of carbohydrate polymers, namely cellulose, hemicellulose, lignin, and a small amount of compounds known as extractives.

Cellulose is an unbranched homopolysaccharide composed of β-D glucose units linked by (1,4) glycosidic bonds. However, the basic building block of cellulose is a dimer of two glucose units known as cellobiose (Fig. 2.2). Cellulose is the most abundant material on Earth, and it is the main constituent of plants. It is also present in bacteria, fungi, algae and even in animals (O'Sullivan, 1997). In nature, cellulose chains have a degree of polymerization (DP) of approximately 10,000 and 15,000 glucopyranose units in wood and native cotton celluloses, respectively (Sjoström, 1981).

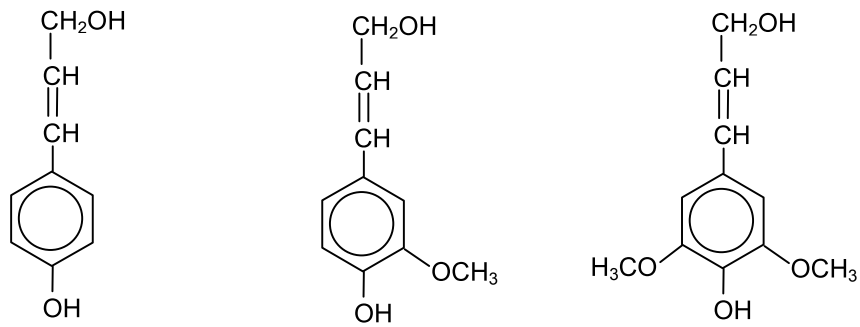
Fig. 2.2. Chemical structure of cellulose.



Cellobiose unit

Hemicellulose or polyose is a mixture of polymers comprising pentoses, hexoses hexuronic acids and deoxy-hexoses. Hemicelluloses differ from celluloses by a composition of various sugar units and by much shorter and branched molecular chains. In contrast to cellulose which is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. Therefore, it is easily hydrolyzed by dilute acid or base, as well as hemicellulase enzymes (Fengel and Wegener, 1989).

Lignin is a complex, hydrophobic, cross-linked, three-dimensional aromatic polymer of phenylpropane building blocks. The mechanical strength properties of plants are mainly due to incorporation of lignin into their cell walls, whereby huge plants such as trees can remain upright. P-coumaryl alcohol (I), coniferyl alcohol (II) and sinapyl alcohol (III) are the primary precursors and building units of all lignins (Fig.2.3).



(I) **(II)** **(III)**

Fig.2.3. The building units of lignin (Fengel and Wegener, 1989).

Lignin is one of the most complicated natural polymers with respect to its structure and heterogeneity, which make it extremely resistant to chemical and biological degradation (Lee, 1997).

The compositional structure of common agricultural residues and wastes is shown in Table 2.1.

Table 2.1. Cellulose, hemicellulose and lignin content in common agricultural residues and wastes.

Agricultural residue	Cellulose	Hemicellulose	Lignin
Hardwood stem	40–50	24–40	18–25
Softwood stem	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Wheat straw	33–40	20–25	15–20
Rice straw	40	18	5.5
Leaves	15–20	80–85	0
Switch grass	30–50	10–40	5–20
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Primary wastewater solids	8–15	NA	24–29
Paper	85–99	0	0–15
Newspaper	40–55	25–40	18–30
Hardwood stem	40–50	24–40	18–25

Adapted from: McKendry (2002), Prasad et al. (2007), Sun and Cheng (2002).

2.4.2. Orange peel

There are a number of different urban and industrial wastes such as cotton linters, cheese whey, residuals of fruit industries, etc which can be used for ethanol production. As a low-cost or even cost-free renewable agricultural residue, conversion of citrus waste to ethanol provides domestic energy supply which can meet a local demand for oxygenating fuel additives while avoiding their disposal-associated problems. The orange is one of the major citrus fruits and its production has increased since the 1980s. Orange production is predicted to approach 66.4 million tons by 2010, representing a 14% increase within 12 years (Plessas et al., 2007). Approximately half of the processed orange is waste consisting of peels, segment membranes and seeds left over after juice extraction (Grohmann and Baldwin, 1992; Wilkins et al., 2007). Nowadays, part of this waste is dried and palletized to be used as a low-value cattle feed, but the drying process is costly due to the high moisture content of the peels, and therefore a large proportion of waste has to be discarded to nature, causing many problems from both economic and environmental points of view (Tripodo et al., 2004). The peel contains various carbohydrate polymers, which make it an interesting choice for production of metabolites such as ethanol by appropriate microorganisms. However, very akin to lignocellulosic materials, individual or combined mechanical, chemical, and biological pretreatment is required to break down cellulose, hemicellulose, and pectin polymers present in the cell walls of orange peels and convert them to their corresponding monomeric sugars (Grohmann and Baldwin, 1992; Grohmann et al., 1994; Grohmann et al., 1995).

Similar to enzymatic hydrolysis of lignocelluloses, the synergic activities of cellulase and β-glucosidase enzymes are required for the cleavage of cellulose and hemicellulose. Besides, pectinase addition is also necessary for complete fractionation of citrus waste. Enzymatic hydrolysis of orange peel produces a mixture of pentose and hexose sugars including arabinose, xylose, glucose, galactose galacturonic acid as well as peel oil. However, presence of peel oil (composed of more than 95% D-limonene), which is extremely toxic to fermenting microorganisms, hinders proper fermentation of orange peel to ethanol. Thus, a successful fermentation usually requires prior separation of D-limonene (hereafter simply called limonene) from the medium, by e.g. filtration or aeration (Grohmann et al., 1994; Lane, 1983; Murdock and Allen, 1960; Wilkins et al., 2007).

3. OVERVIEW OF ETHANOL PROCESS

Several cellulose-based ethanol-producing plants were constructed during World War II, when wartime conditions changed the economic conditions and priorities. Many of these plants after the war could not stay viable in competition with the synthetically produced ethanol. In the following decades, interest in ethanol as a fuel to some extent was dependent on the price of oil. However, shortage of oil supplies, the growing desire for cuts in the net emission of CO_2 and global warming together with rising demand for human food and feed have maintained research and development on the ethanol production from celluloses as cheap and renewable raw materials. Many countries have initiated extensive programs in biofuel as a sustainable energy resource for displacement of transportation fuels (Table 3.1) (Himmel et al., 2007). By further decrease in the cost of enzymes for hydrolysis, and modern technology available today for new ethanol plants, the second generation of ethanol, that is, cellulosic-based ethanol, will gain the potential to compete on a large scale with gasoline without subsidies in the next decade (Goldemberg, 2007; Solomon et al., 2007).

Table 3.1. Cellulosic ethanol pilot and commercial plants under construction.

Company	Location	Feedstock	Capacity or feed rate	Start date
Pilot plants				
Iogen	Ottawa, Canada	Wood chips	9.0×10^2 kg day^{-1}	1985
Iogen	Ottawa, Canada	Wheat straw	9.0×10^2 kg day^{-1}	1993
Masada/TVA	Muscle Shoals, AL	Wood	NA	1993
SunOpta	Norval, Canada	Various (non-woody)	4.5×10^2 kg h^{-1}	1995
Arkenol	Orange, CA	Various	9.0×10^2 kg day^{-1}	1995
NREL/DOE	Golden, CO	Corn stover, others	9.0×10^2 kg day^{-1}	2001
Pearson Technologies	Aberdeen, MS	Wood residues, rice straw	27 Mg day^{-1}	2001
NEDO	Izumi, Japan	Wood chips	3.0×10^2 l day^{-1}	2002
Dedini	Pirassununga, Brazil	Bagasse	1600 m^3 year^{-1}	2002
Tsukishima Kikai Co.	Ichikawa, Chiba, Japan	Wood residues	9.0×10^2 kg d^{-1}	2003
PureVision	Ft. Lupton, CO	Corn stover, bagasse	9.0×10 kg day^{-1}	2004
Universal Entech	Phoenix, AZ	Municipal garbage	1.0×10^2 l day^{-1}	2004
Sicco A/S	Odense, Denmark	Wheat straw	1.0×10^2 kg h^{-1}	2005
Abengoa Bioenergy	York, NE	Corn stover (co-located with grain ethanol plant)	2000 m^3 year^{-1}	2006
Demonstration Plants				
Iogen	Ottawa, Canada	Wheat, oat and barley straw	3000 m^3 year^{-1}	2004
ClearFuels Technology	Kauai, HI	Bagasse and wood residues	11,400 m^3 year^{-1}	2007
Celunol	Jennings, LA	Bagasse, rice hulls (co-located with grain ethanol plant)	5000 m^3 year^{-1}	2007
SEKAB	Sweden	Softwood residues (spruce, pine)	30,000 m^3 year^{-1}	2009

Adapted from: Solomon et al. (2007).

The technology of ethanol production from biomass feedstocks consists of several steps, and varies depending on the type of raw materials used. It becomes more sophisticated as the raw materials turn from sugars to starches and cellulosic materials. Unlike starch, the specific structure of cellulose favors the ordering of the polymer chains into tightly packed, highly crystalline structures that are water-insoluble and resistant to depolymerization. For production of ethanol from cellulosic feedstocks, four major unit operations are required: pretreatment, hydrolysis, fermentation, and separation/ purification (Mosier et al., 2005; Solomon et al., 2007; Taherzadeh and Karimi, 2007).

3.1. Pretreatment

One of the main problems in application of lignocellulosic materials is their resistance against enzymatic depolymerization. The carbohydrate polymers in lignocellulose are tightly bound to lignin mainly by hydrogen bonds as well as by some covalent bonds which make it a recalcitrant substrate for hydrolysis and ethanol production. Thus, delignification is a crucial step prior to depolymerization and fermentation steps which can highly increase the rate of subsequent hydrolysis reaction. Delignification liberates cellulose and hemicellulose from their complex with lignin (Duff and Murray, 1996; Lin and Tanaka, 2006; Szczodrak and Fiedurek, 1996). Citrus wastes are more susceptible to enzymatic hydrolysis, probably due to absence of lignin in their structure. However, presence of cellulose, hemicelloluse and pectin polymers bound to each other in three-dimensional structures makes them relatively resistant materials for hydrolysis (Grohmann et al., 1995).

Table 3.2. Common pretreatment methods for lignocellulosic materials.

Method	Effects
Physical	
Mechanical comminution (milling and grinding), Pyrolysis	Increase in specific surface area and size of pores, decrease of the degrees of polymerization of cellulose and its crystallinity, decomposition of cellulose
Physico-Chemical	
Steam explosion, Ammonia fiber explosion (AFEX), CO_2 explosion	Partial degradation of hemicellulose, increase of the specific surface area and size of pores, lignin transformation
Chemical	
Ozonolysis, Acid hydrolysis, Alkaline hydrolysis, oxidative delignification, organic solvents	Hemicellulose degradation, lignin removal, increase in internal surface area due to swelling, decrease in degree of polymerization and crystallinity, increase in porosity
Biological	
Brown-, white- and soft-rot fungi	Hemicellulose and lignin degradation, increase of the specific surface area and size of pores

Adapted from: Duff and Murray (1996), McKendry (2002), Prasad et al. (2007), Sun and Cheng (2002), Szczodrak and Fiedurek (1996).

The objective of pretreatment is therefore to increase the surface area and porosity of the substrate, reduce the crystallinity of cellulose and disrupt the heterogeneous structure of cellulosic materials. This process makes the carbohydrate polymers accessible for depolymerization. Pretreatment and hydrolysis of lignocellulosic materials result in a number of fermentable pentose and hexose sugars, leaving lignin as a by-product which can be used as fuel to produce heat or electricity. Various methods for pretreatment of lignocellulosic materials can be classified in four major groups, as summarized in Table 3.2.

3.2. Hydrolysis

Hydrolysis of cellulosic materials includes the processing steps that convert the carbohydrate polymers e.g. cellulose and hemicellulose into monomeric sugars. Cleavage of these polymers can be catalyzed enzymatically by cellulases or chemically by acids such as sulfuric acid (Mosier et al., 2005). The factors that have been identified to affect the hydrolysis of cellulosic biomass include porosity or accessible surface area, cellulose fiber crystallinity, and the content of lignin and hemicellulose (Prasad et al., 2007).

3.2.1. Enzymatic hydrolysis

Hydrolysis of cellulosic materials can be catalyzed by a class of enzymes known as cellulases. These enzymes are mainly produced by fungi, bacteria, and protozoans that catalyze the cellulolysis or hydrolysis of cellulose. At least three major groups of enzymes including exo-glucanase, endo-glucanase and β-glucosidase are involved in depolymerization of cellulose to glucose. β-glucosidase catalyzes cleavage of cellobiose, which plays a significant role in the hydrolysis process, since cellobiose is an end-product inhibitor of many cellulases including both exo- and endo-glucanases (Galbe and Zacchi, 2002; Lee, 1997; Rabinovich et al., 2002; Sun and Cheng, 2002). β-glucosidase, in turn, is inhibited by glucose and, therefore, enzymatic hydrolysis is sensitive to the substrate concentration (Philippidis et al., 1993). In addition to substrate concentration, pretreatment of cellulosic materials and hydrolyzing conditions such as temperature and pH are among factors influencing the effectivity of enzymatic hydrolysis. Most cellulase enzymes show an optimum activity at temperatures and pH in the range of 45-55°C and 4-5, respectively (Duff and Murray, 1996; Galbe and Zacchi, 2002).

The enzymatic hydrolysis process can be accomplished using different strategies, of which the most important ones include separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In SHF, hydrolysis and fermentation are carried out in separate vessels under their own optimal conditions; however, end-product inhibition of enzymes' activity and contamination problems are associated with this process. In order to eliminate drawbacks of the SHF process, SSF that combines hydrolysis and fermentation in one vessel has been developed. Sugars produced during hydrolysis are immediately fermented into ethanol and thus, problems associated with sugar accumulation and enzyme inhibition as well as contamination can be avoided (Galbe and Zacchi, 2002; Ohgren et al., 2007; Philippidis et al., 1993; Wyman et al., 1992). The main drawback of SSF is the different optimum temperatures of the hydrolysis and fermentation processes. Most fermenting yeasts have an optimal temperature around 30-35 ºC while hydrolyzing enzymes show optimal activities around 50 ºC (Kadar et al., 2004).

3.2.2. Acid hydrolysis

Interest in wood hydrolysis dates back to 1819 when Braconnot discovered that cellulose could be dissolved in concentrated acid solutions and converted to sugar. Acid hydrolysis can be performed with various types of acids including sulfuric, sulfurous, hydrochloric, phosphoric, nitric acid, etc. Acid hydrolysis is subdivided into concentrated- and dilute-acid hydrolysis. Through the concentrated-acid hydrolysis, the biomass is treated with high concentration of acids at near ambient temperatures, which results in high yield of sugars. However, this process has drawbacks including high acid and energy consumption, equipment corrosion and longer reaction time (Galbe and Zacchi, 2002; Harris et al., 1945; Taherzadeh and Karimi, 2007).

Dilute-acid hydrolysis, on the other hand, uses low-concentration acids e.g. 0.5-1% H_2SO_4 and high temperatures. High temperature is required to attain acceptable rates of cellulose conversion to glucose. Despite low acid consumption and short reaction time in dilute-acid hydrolysis, application of high temperatures in this method accelerates the rate of sugar decomposition and increases equipment corrosion (Galbe and Zacchi, 2002; Taherzadeh and Karimi, 2007). Decomposition of sugars not only lowers the ultimate yield of sugars in dilute-acid process, but also produces a number of by-products that show severe inhibiting effects on the subsequent fermentation step (Klinke et al., 2004; Luo et al., 2002; Taherzadeh et al., 1997a). Although many of these inhibitors have been identified and their effects on fermentation either individually or in combination have been vastly investigated using synthetic medium (Delgenes et al., 1996; Martin and Jönsson, 2003; Taherzadeh et al., 1999a; Taherzadeh et al., 2000; Taherzadeh et al., 1997b), the composition of dilute-acid hydrolysis remains far more complex than these synthetically made media. Thus, a synthetic medium containing even all known inhibitors similar to a sample of dilute-acid hydrolyzate may show different results in the fermentation step. Nevertheless, parallel investigation on both synthetic and dilute-acid hydrolyzates for many years has provided a great extent of valuable information regarding potential effects of these toxic compounds on baker’s yeast. Based on this information, the main inhibiting compounds are classified in three groups: furans, phenolic compounds, and carboxylic acids (Clark and Mackie, 1984).

In order to decrease the amount of sugar degradation and consequently formation of inhibitors, a two-stage process has been developed where hemicellulose sugars are released in the first stage under milder conditions. The solid residue, mainly consisting of cellulose, is then separated from the liquid hydrolyzate and subjected to the second-stage hydrolysis which is performed under harsher conditions. A range of temperatures between 170-190°C in the first stage and 200-230°C in the second stage is commonly used (Galbe and Zacchi, 2002).

Although enzymatic hydrolysis is an efficient method to release almost all carbohydrates present in the orange peels, its application is hindered by the high cost of enzymes and the slow rate of the depolymerization reaction. The advantages of dilute-acid hydrolysis for peel liquefaction and releasing carbohydrates prior to enzymatic treatment have already been studied (Grohmann et al., 1995; Vaccarino et al., 1989). Dilute-acid hydrolysis is an efficient pretreatment which can solubilize a large fraction of citrus peel, leading to substantial reduction of enzyme consumption. However, glycosidic bonds between units of pectin are extremely resistant to this treatment. Therefore a cost-effective method through which all or a high proportion of carbohydrates could be released is required. This method can be a combination of both acid and enzymatic hydrolysis where the variables influencing dilute-acid hydrolysis are set to yield maximum solubilization of citrus peel. The solid residual could be further hydrolyzed by a mixture of required enzymes.

When a process is affected by several variables with possible interaction effects, statistical approaches in experimental design provide a powerful tool to study them simultaneously and find the optimal conditions. This approach was exploited to optimize the dilute-acid hydrolysis of orange peel for maximum solubilization of sugars (paper I).

3.2.2.1. Formation of inhibitory substances

As described, monomeric sugars are not the only products of dilute-acid hydrolysis, since liberated sugars undergo secondary reactions and are converted to other chemical compounds which are mostly toxic for fermenting microorganisms (Klinke et al., 2004; Larsson et al., 1999) . The simple pathway of side reactions from lignin and liberated sugars (both pentoses and hexoses) to their corresponding inhibitory chemical compounds is presented in Fig. 3.1.

Monosaccharides, especially those produced in the early stage of dilute-acid hydrolysis (mostly from hemicellulose), are decomposed to furan derivatives, namely furfural and HMF. The formation of furfural and HMF from lignocellulosic materials is a first-order reaction (Rodriguez-Chong et al., 2004; Saeman, 1945). The furan derivatives can be further converted to formic acid and levulinic acid. Acetyl group in hemicellulose through the hydrolysis can result in acetic acid in the resultant hydrolyzate. These weak acids with relatively high pK_a at the higher concentrations have negative impact on the cell viability, even though their toxicity is pH-dependent. Undissociated forms of these acids can penetrate through the plasma membrane and dissociate in the cytoplasm and disturb neutral intracellular pH. The cell pumps out the surplus H^+ through the action of plasma membrane ATPase in order to maintain its intracellular pH, which can lead to lower biomass yield and even cell death at high acid concentration (Pampulha and Loureirodias, 1989; Taherzadeh et al., 1997b; Verduyn et al., 1990a).

Furfural and hydroxymethylfurfural (HMF) are known as two of the strongest inhibitor compounds present in the hydrolyzate. A correlation between specific ethanol production rate by *S. cerevisiae* in dilute-acid hydrolyzate and the sum of furfural and HMF concentrations has been reported (Taherzadeh et al., 1997a). Higher concentrations of furfural and HMF resulted in less fermentability of the hydrolyzates. Furfural is metabolized by *S. cerevisiae* and reduced to corresponding alcohol (furfuryl alcohol). This conversion is mediated by NADH-dependent alcohol dehydrogenase (ADH). Presence of furfural in the cultivation medium could result in reduced biomass yield, decrease in specific growth rate and ethanol productivity (Azhar et al., 1981; Boyer et al., 1992; Taherzadeh et al., 1999a). HMF is also metabolized by yeast but at a lower rate than furfural, imposing longer lag phase in growth (Taherzadeh et al., 2000).

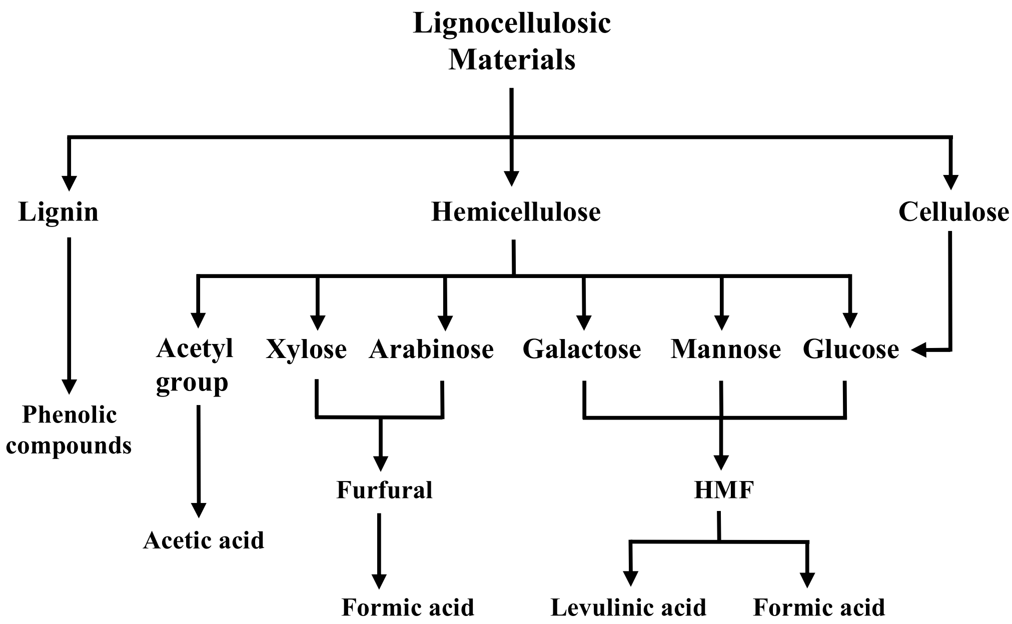


Fig. 3.1. Main inhibitory compounds formed during dilute-acid hydrolysis.

Formation of Furfural and HMF during dilute-acid hydrolysis is a sequential reaction where cellulose and hemicellulose first are hydrolyzed to their monomers, followed by further degradation to furan derivatives. The kinetics of these two reactions were studied and the results indicated that these hydrolysis and decomposition reactions are both first-order reactions and possess rates of similar magnitude (Saeman, 1945). These two reaction rates are influenced by temperature and acid concentration. The profile of total liberated carbohydrates vs. time passes through a maximum, and hence there is an optimal time at which sugar production reaches a maximum. This time is a function of constant values of both reactions. Elapsing time of hydrolysis longer than the optimal value enhances the speed of the second reaction, leading to a decrease in net total sugar liberation (Saeman, 1945).

Phenolic compounds mostly originate from lignin decomposition and to a minor extent from the aromatic wood extractives (Taherzadeh and Karimi, 2007). Inhibition mechanisms of phenolic compounds on *S. cerevisiae* and other eukaryotic microorganisms have not yet been completely elucidated, largely due to the heterogeneity of the group and the lack of accurate qualitative and quantitative analyses. Phenolic compounds may partition into biological membranes, causing loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices (Almeida et al., 2007; Palmqvist and Hahn-Hägerdal, 2000). Low molecular weight (MW) phenolic compounds are more inhibitory to *S. cerevisiae* than high MW compounds. Treatment of hydrolyzate with laccase, a lignin-oxidizing enzyme, resulted in less inhibition of fermentation (Jönsson et al., 1998).

3.2.2.2. Dilute-acid pretreatment of orange peel

The advantages of dilute-acid hydrolysis for peel liquefaction and releasing carbohydrates prior to enzymatic treatment have already been studied by several researchers (Grohmann et al., 1995; Vaccarino et al., 1989). Dilute-acid hydrolysis of citrus peel is affected by several variables including temperature, acid concentration (or pH), total solid fraction (TS), and time duration of the hydrolysis.

An experimental design was performed to find the optimal conditions and the main and interactive effects of variables (paper I). The objective was to maximize the yield of total sugars (1^{st} response variable) and minimize the yield of HMF (2^{nd} response variable).The results of statistical analysis showed that temperature and acid concentration in linear and quadratic form were highly significant for the yield of total sugars ($p \leq 0.05$). Among the interactive terms, only interaction between temperature and acid concentration was highly significant. For the second response variable (Y_{HMF}), temperature and acid concentration had the greatest effect on the formation of HMF, followed by total solid concentration and time.

A typical 3-D response surface plot with the corresponding contour plot for the yield of total carbohydrates is depicted in Fig. 3.2 a,b. These plots show the effects of temperature and acid concentration on the yield of sugars, when solid fraction and time were selected at their center points. At the lower levels of temperature, increase in acid concentration resulted in higher yield of sugars. Similarly, at low levels of acid concentration, increase in temperature had a positive effect on the sugar yield. However, at the higher levels of both temperature and acid concentration, the yield of sugars declined, certainly due to presence of the strong interaction between these two variables (paper I). The region of optimum was around 117°C and 0.6% acid concentration where the maximum yield of sugars, 42.6% (g sugars/g peel dry mass), is attainable.

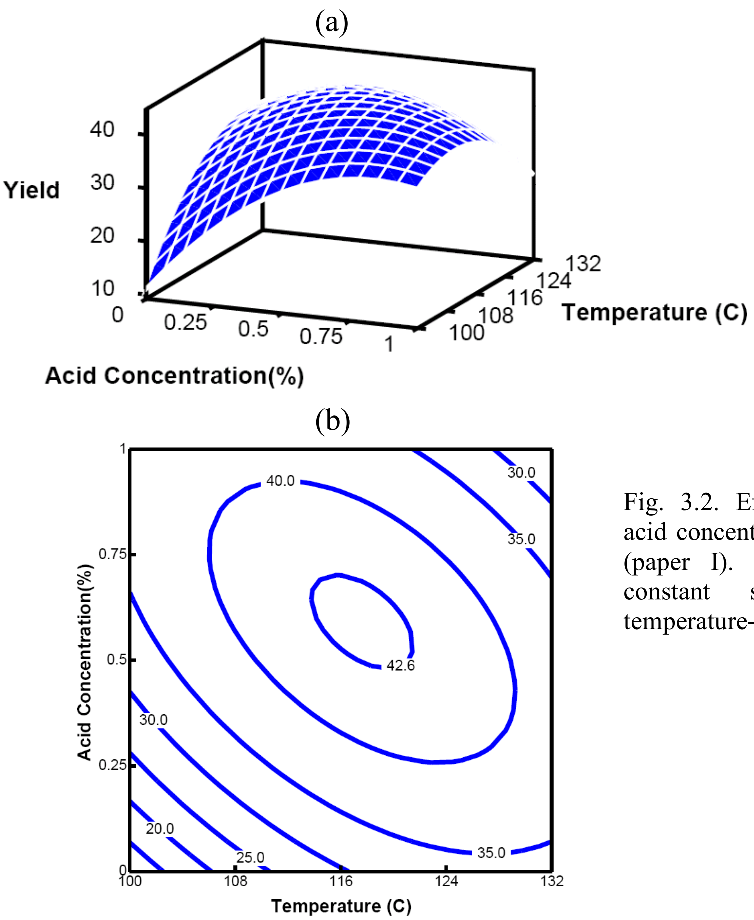


Fig. 3.2. Effect of temperature and acid concentration on the sugars yield (paper I). Lines in (b) show the constant sugar yield in the temperature-acid concentration plane.

3.3. Fermentation

3.3.1. Microorganisms

Microorganisms play a significant role in production of ethanol from renewable resources and thus, selection of suitable strain is essential for the individual process. Ethanol production is much more challenging and difficult when lignocellulosic and/or cellulosic materials are to be used as raw materials. Unlike the starch-based materials, pretreatment and hydrolysis of lignocellulosic materials produce a mixture of pentoses and hexoses along with other inhibiting compounds, causing many problems in the fermentation step. Therefore, capability of consuming both pentose and hexose sugars, high tolerance against substrate, ethanol as well as inhibiting compounds, high ethanol yield and minimum nutrient requirements are the essential features of an ideal microorganism (van Zyl et al., 2007). Although no microorganism has been found yet to meet all these requirements, development of a desirable strain is the focus of many studies. Thus far, wide varieties of microorganisms including yeasts, bacteria and fungi have been exploited offering different advantages and disadvantages (Olsson and Hahn-Hägerdal, 1993). However, the most frequently used microbe has been yeast and among the yeasts, *S. cerevisiae* which can tolerate ethanol concentration as high as ca. 20% of fermentation medium is the preferred strain (Lin and Tanaka, 2006). Some species of bacteria such as *Zymomonas mobilis* and the genetically engineered *Escherichia coli* can produce ethanol at higher yields, but they are less resistant to the end product (ethanol) and other compounds present in the hydrolyzates when compared to the yeast (Lawford and Rousseau, 1998; Olsson and Hahn-Hägerdal, 1993; Sprenger, 1996).

During the evolution of yeast *S. cerevisiae*, the ability to override the glucose repression circuit that suppresses respiration of glucose and other hexose sugars above 20-40 mM threshold concentration in the presence of oxygen was developed. This phenomenon, known as the 'Crabtree effect', provided the ancestor of *S. cerevisiae* with an advantage over its competitors because high ethanol levels (>4% v/v) are toxic to most other microorganisms (van Zyl et al., 2007; Verstrepen et al., 2004). In contrast with many advantages offered by using yeast in ethanol production, it lacks the mechanism to take up pentose sugars as substrate. Attempts to add this ability by genetic manipulation are still at the laboratory stage (Jeffries, 2006). Putting advantages and disadvantages together, *S. cerevisiae* still remains the prime microorganism for ethanol production (Bai et al., 2008).

3.3.2. Fermentation strategies

A sugar such as glucose is directly metabolized by the yeast cells through the glycolysis pathway to gain energy for biosynthesis. Under anaerobic conditions, the overall reactions produce two moles of ethanol and CO_2 per mole of glucose consumed. Fermentation can be carried out with different types of industrial operation as batch, fed-batch, or continuous process. The most suitable choice depends on the kinetic properties of the microorganism as well as process economics. Batch cultivations need low investment cost and lower requirements for process control. The fed-batch operation, sometimes regarded as a combination of the batch and continuous operations, involves addition of feed at constant intervals while effluent is removed discontinuously. When the substrate has inhibitory effects, this method is advantageous because the microorganism is exposed to low concentration of substrate. Continuous operation offers ease of control and high ethanol productivity, but contamination is a serious issue to be considered (Olsson and Hahn-Hägerdal, 1996; Prasad et al., 2007).

Ethanol production from biomass can be carried out by using free or immobilized cells. The natural ability of many microorganisms to adhere to different kinds of surfaces is well known. Many microbial cells grow and aggregate within or on the surfaces of natural structures. Whole cell immobilization is defined as "the physical confinement or localization of intact cells to a certain region of space with preservation of some desired activity" and shares the same feature (Karel et al., 1985). Immobilization puts the cells in close contact together and forces them to aggregate. The resultant cell community can obtain much better protection from harsh environmental conditions. Immobilization is an efficient method for cell retention, and the cells show better tolerance in severely inhibiting medium than those from other methods such as cell filtration and recirculation (Bai et al., 2008; Brandberg et al., 2007). Immobilized cell system is also attractive due to other advantages including a better protection against shear force, improved cell stability, and decreased cost of recovery, recycling and downstream processing. Furthermore, continuous processes using immobilized cells can be operated at rather high dilution rates, which leads to higher volumetric productivity and shorter residence times (Park and Chang, 2000; Purwadi and Taherzadeh, 2008; Verbelen et al., 2006).

A number of cell immobilization methods have been developed for different applications. These methods are mainly divided into four categories based on the physical mechanism of cell localization and the nature of support: (a) attachment to a solid surface, (b) entrapment within a porous matrix, (c) self-aggregation by natural flocculation or with artificial cross-linking agents, and (d) cell containment behind a barrier or encapsulation (Kourkoutas et al., 2004; Verbelen et al., 2006).

3.4. Cell encapsulation

The idea of using polymer membrane microcapsules was first reported by Chang (1964) for immunoprotection of transplanted cells. He introduced the term of "artificial cells" for biologically active materials enclosed in a semipermeable membrane. The cells are surrounded by a biocompatible matrix with sufficient mechanical strength that permits the entry of small molecules such as essential nutrients and oxygen and the exit of toxic metabolites and hormones while excluding antibodies and other immunologic moieties and as a result, encapsulated cell can be protected from destruction by foreign invaders (Bhatia et al., 2005; Orive et al., 2004). Since then, cell encapsulation continues to hold significant promise for various applications in biotechnology, food industry and medicine. Depending on the type of application, materials can be encapsulated for the purpose of isolation, purification or slow release. The coating material, also called a capsule, membrane, carrier or shell, is generally composed of natural or synthetic polymers such as polysaccharides (natural or modified), proteins, lipids, polyamides, nylon, etc. The main reason for using polymers for encapsulation lies in their ability to exist at different phases as liquids, gels or solids, which enables them to meet a large range of mechanical and physical demands (Gibbs et al., 1999; Kampf, 2002; Park and Chang, 2000). In addition to biocompability of polymers used for encapsulation, they must satisfy several other requirements, some shared with the other immobilization techniques (Jen et al., 1996).

The membrane is formed by gelation or solidification of some polymers, occurring through a change in temperature for thermoreversible gels, or via chemical or ionic crosslinking or formation of an insoluble complex (Bhatia et al., 2005). Due to the absence of a solid or gelled core and small membrane thickness, mass transfer resistance is less than that of the entrapment method. The membrane of the capsule should be designed to have proper permeability allowing sufficient transport of the required nutrients and cell products across the network (Groboillot et al., 1994; Jen et al., 1996). The major disadvantages of encapsulation are ultimate mass transfer limitations and possible membrane fouling caused by cell growth (Lebeau et al., 1998). Biocompatibility, mechanical and chemical stability of coating shell in physiological environment, production of uniform capsules with excellent repeatability and reproducibility are among other challenges that must be addressed for individual application. The adoption of automated machines for microencapsulation could result in improved reproducibility in terms of shape, size and morphology (Orive et al., 2003).

Polyelectrolytes that can form precipitate and gels either through complexation with an oppositely charged polyion, or ionic crosslinking with a multivalent counterion have been widely used for microencapsulation of cells. Alginate is the most frequently studied material due to its biocompatibility, ease of formulation, mild gelation conditions and acceptability as a food additive (DeGroot and Neufeld, 2001). Alginate is a copolymer of mannuronic acid (M block) and guluronic acid (G block), arranged in long sequences containing either all M units, all G units, or alternating M and G units. Inter-chain linkage between G blocks which is promoted by divalent cations such as Ca^{+2} and Ba^{+2} forms a three-dimensional gel network (Fig. 3.3).

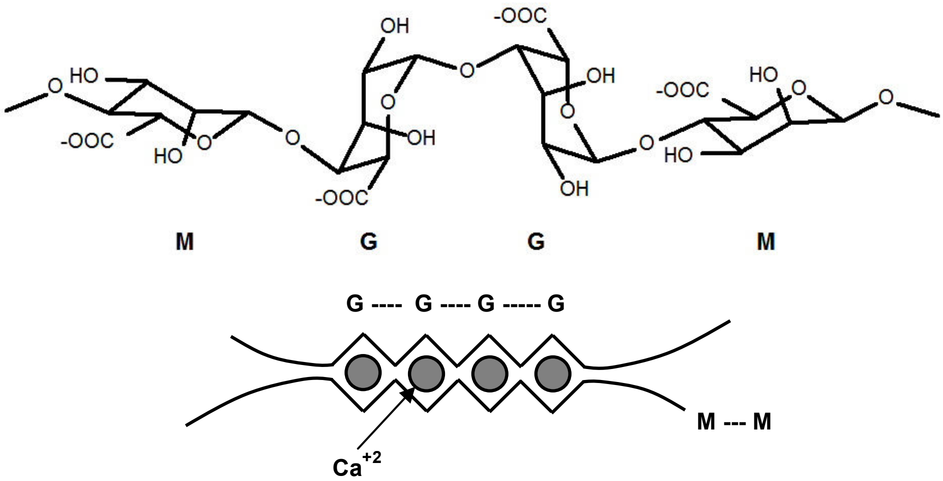


Fig. 3.3. Inter-chain linkage between G blocks and Ca^{+2}.

Several techniques for cell encapsulation have been developed, among which the more important methods – coacervation, interfacial polymerization, pregel dissolving and liquid droplet formation – are described in the following.

3.4.1. Coacervation

This method, also called coextrusion, is based on dispensing the cell suspension through the inner needle and the polymeric solution through the outer needle of a concentric needle assembly. The liquid core (cell suspension) is wrapped with a uniform layer of polymeric solution which can be solidified by means of heat, crosslinking, or solvent removal techniques (Kampf, 2002; Park and Chang, 2000).

3.4.2. Emulsion – Interfacial polymerization

Interfacial polymerization occurs between monomers dissolved in the respective immiscible phases. An aqueous solution containing bioactive material and the water-soluble monomer is emulsified in the organic phase by stirring. The capsule membrane is then formed by addition of the other organic solvent-soluble monomer to the continuous phase. This method enables the preparation of thin, mechanically stable capsules with easy control over the size and permeability (Green et al., 1996; Park and Chang, 2000).

3.4.3. Pregel dissolving

In this technique, the calcium alginate beads containing cells are first made using the conventional method. Then, the carboxyl group of calcium alginate bead reacts with acid-reactive amine or imine group in the polylysine or polyethylenimine to form a polyelectrolyte complex membrane on the surface of the bead. Next, the gel core is dissolved in a sodium citrate solution to obtain liquid core capsules (Lim and Sun, 1980).

3.4.4. Liquid droplet formation

A reverse procedure of the formation of conventional calcium alginate beads is applied to make capsules through a one-step method. In this method, a solution of $CaCl_2$ containing microbial cells is dropwise added into a swirling sodium alginate solution, where the membrane is formed immediately on the surface of the aqueous droplet through the ionic interaction between calcium and alginate. Thickness and pore size of the membrane, surface charge and mechanical strength of the capsules can be easily controlled by alteration of alginate, calcium and gel-forming polymer (Klein et al., 1983; Park and Chang, 2000).

3.5. Ethanol production using immobilized yeast cells

High cell concentration in the cultivation media can help to decrease the fermentation time as well as increasing the productivity of fermentation. Besides, high cell concentration in media containing toxic compounds such as wood hydrolyzates is a strategy to improve the tolerance of the cells against the inhibitors (Chung and Lee, 1985; Galazzo and Bailey, 1990). Although encapsulated yeast cells have been rarely used in ethanol production, other immobilizing techniques such as cell entrapment are frequently reported. Yeast cell immobilization using various support materials is an efficient method which can provide high cell density in the bioreactor. Even though yeast is known as a highly tolerant strain to the ethanol in the medium, using an immobilized yeast cell system improves this tolerance even more, allowing the fermentation of substrate with high sugar concentration (Jirků, 1999b). Various bioreactor configurations are being used for fermentation with immobilized yeast, including the packed-bed reactor, fluidized-bed reactor, bubble column and stirred tank reactor. The latter provides increased mass transfer rate by means of forced agitation, but more stable carrier support is required due to high shear stress (Verbelen et al., 2006).

While ethanol production by *S. cerevisiae* free cells is pH-dependent, using a column reactor packed with entrapped yeast beads was shown to be independent of pH in the range of 3.5-6.5 (Jirků, 1999a). Nigam (2000) reported that the volumetric ethanol productivity of immobilized *S. cerevisiae* in packed-bed column reactor was 42.8 g/l·h at dilution rate of 1.5 h^{-1}. This value was ca. 11.5 times higher than the corresponding value for free-cell system. Ethanol productivity of encapsulated *S. cerevisiae* during the sequential batch cultivations reached a maximum rate of 66.4 g/l·h which was seven times greater than that found in free-cell culture (Mei and Yao, 2002). Furthermore, when toxic compounds with hydrophobic nature such as peel oil are present in fermentation medium, utilization of a membrane with hydrophilic characteristic could act as a selective barrier that excludes the oil and protects the cells from direct contact with toxic compound. Thus, the fermentation can be carried out regardless of the concentration of such a toxic compound in the medium.

4. METHODOLOGY

4.1. Microorganisms

The yeast *S. cerevisiae* CBS 8066, obtained from Centraalbureau voor Schimmelcultures (Delft, The Netherlands), was used in all experiments throughout this work. The strain was maintained on agar plates made from yeast extract 10 g/l, soy peptone 20 g/l, and agar 20 g/l with D-glucose 20 g/l as an additional carbon source. A defined synthetic growth medium was used with the following chemical compositions per liter of solution: 7.5 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4, 0.75 g $MgSO_4.7H_2O$, 30 mg EDTA, 13 mg $CaCl_2.6H_2O$, 9 mg $ZnSO_4.7H_2O$, 6 mg $FeSO_4.7H_2O$, 2 mg H_3BO_3, 2 mg $MnCl_2.4H_2O$, 0.8 mg $Na_2MoO_4.2H_2O$, 0.6 mg $CoCl_2.2H_2O$, 0.6 mg $CuSO_4.5H_2O$, 0.2 mg KI, 50 µg *d*-biotin, 0.2 mg p-aminobenzoic acid, 1.0 mg nicotinic acid, 1.0 mg calcium pantothenate, 1.0 mg pyridoxine hydrochloride, 1.0 mg thiamine hydrochloride, 25 mg *m*-inositol, 0.5 ml antifoam, 10 mg ergosterol and 420 mg Tween 80.

4.2. Pretreatment of orange peel, optimization

In the present work, the central composite rotatable design (CCRD) was applied to evaluate the main and interactive effects of four variables on the yields of total liberated sugars and formation of hydroxymethylfurfural (HMF) in dilute-acid hydrolysis of orange peel (Montgomery D. C. et al., 2001; Obeng et al., 2005). Based on the experimental design, a pair of second-order model was developed and the optimum conditions to attain simultaneously the highest yield of carbohydrates and lowest yield of HMF were predicted and validated by an additional experiment (paper I). A software package, MINITAB®, was used to evaluate the experimental results and to fit them to the model according to the following equation:

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^{\,2} + \sum_{i}^{i \prec j} \sum_{j} b_{ij} x_i x_j + e \qquad (4.1)$$

where Y is the dependent (or response) variable(s) to be modeled, x_i and x_j are the independent variables (factors), and b_i, b_{ii} and b_{ij} are the measures of the x_i , x_i^2 and x_ix_j effects, respectively. The variable x_ix_j represents the first-order interactions between x_i and x_j, and e is the error. When the response data are obtained from the experiments, a regression analysis using the least-squares method is performed to determine the coefficients of the approximating polynomials or response model, their standard errors, and significance of variables and their interactions. The predicted response variables and the optimum region of the selected variables are obtained from 3-D surface plots, corresponding contour plots and MINITAB® optimizer.

4.3. Media

Dilute-acid hydrolysis of peels, previously ground with a food homogenizer (ULTRA-TURAX,TP 18-20, Janke & Kunkel Ika-Labortechnik,Germany) were carried out at various solid fractions (paper I). Sulfuric acid (98%) was added to the 100 ml peel/water slurry to reach final acid concentration of 0, 0.25, 0.5, 0.75, and 1% v/v. Next, the slurries were heated at various temperatures of 100, 108, 116, 124, and 132°C with different residence times of 5, 10, 15, 20, and 25 min according to the experimental design (paper I).

The media used in the current work were defined synthetic medium with the compositions as previously described, in papers II, III, and IV, dilute-acid wood hydrolyzate stages 1 and/or 2 in papers II and III. Furthermore, orange peel hydrolyzate was used in paper V.

The wood hydrolyzates used in papers II and III were produced in a two-stage dilute-acid hydrolysis of Swedish forest residuals, originating mainly from spruce trees. The wood chips hydrolysis was carried out in a 350-L rebuilt masonite gun batch reactor located in Rundvik (Sweden). Dry wood chips were impregnated in 0.5% H_2SO_4 solution to make a final solid concentration of 30% w/w. The mixture was loaded into the reactor where direct steam was injected to heat up the contents of the reactor for a period of about 50 seconds. The first stage of hydrolysis was performed at 12 bar (188˚C) for 7–10 min followed by a rapid decompression and discharge of the material into a collecting vessel. The solid residue was then separated from the liquid hydrolyzate by filtration. The second-stage hydrolysis of remaining solid was carried out at a pressure of 21 bar (215˚C) for 7 min. The compositions of the hydrolyzates are presented in Table 4.1.

Table 4.1. Composition of dilute-acid hydrolyzate stages 1 and 2.

Components concentration (g/l)	Paper I Stage 1	Paper I Stage 2	Paper II Stage 2
Glucose	6.38	19.86	19.30
Mannose	14.55	2.80	6.55
Xylose	7.68	2.22	3.08
Galactose	3.36	0.10	0.62
Furfural	0.39	0.31	0.37
HMF	0.74	1.58	1.35
Acetic acid	4.95	1.35	2.50

The peels used in paper IV were the residuals of Argentina Orange obtained from Brämhults juice factory (Borås, Sweden) which were milled and then enzymatically hydrolyzed by a mixture of three commercial enzymes, Pectinase (Pectinex Ultra SP), Cellulase (Celluclast 1.5 L) and β-glucosidase (Novozym 188) provided by Novozymes A/S (Bagsvaerd, Denmark). Hydrolysis was carried out at 45˚C for 24 h with 12% solid concentration. The sugar and sugar acid fraction of dry orange peel hydrolyzate was: glucose 22.9% (±2.4), fructose 14.1% (±1.3), galactose 4% (±0.2), arabinose 7.1%

(±0.5), xylose 0.4% (±0.1), galacturonic acid 19% (±1.7). Concentration of limonene in hydrolyzate was 0.52% v/v.

4.4. Encapsulation procedure

Among several techniques available for cell encapsulation, the *liquid-droplet-forming, one-step method* was used and further developed in the current work. In this method, the inoculum's cells were centrifuged and re-suspended in 1.3% $CaCl_2$ solution containing 1.3% carboxymethylcellulose (CMC) and the mixture was thoroughly mixed. This solution was added drop-wise through an extruder with 8 needles into 0.6% sterile sodium alginate solution containing 0.1% Tween 20 to create the capsules with mean diameter of 3.9–4.2 mm and 0.17 (±0.02) mm in membrane thickness. CMC was added to increase the viscosity of the first solution to form spherical capsules, and Tween 20 was used as a surfactant to improve the permeability of the capsule membrane. The capsules were then washed with distilled water and hardened in 1.3% $CaCl_2$ solution for minimum 30 min. The resultant capsules were harvested and cultivated in a synthetic growth medium to prepare high biomass concentration inside the capsules. Schematic illustration for encapsulation procedure is depicted in Fig 4.1.

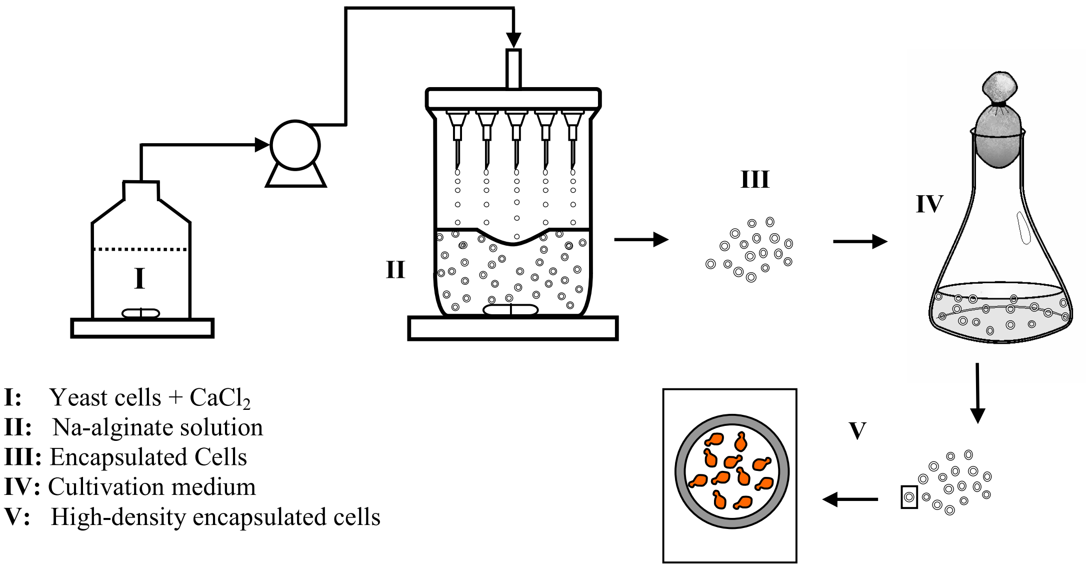


Fig.4.1. Schematic method of cell encapsulation.

4.4.1. Further development of capsules

The mechanical strength of the capsules is of great importance, especially when they are exposed to the high shear stress in bioreactors. The ionic interaction between alginate and Ca^{+2} is weakened in physiological environment or in common buffer solutions with high concentration of citrate and phosphate ions, which can lead to loss of membrane integrity (Taqieddin and Amiji, 2004). The stability of alginate capsules can be improved by coating them with an extra layer of polyelectrolytes which can interact with alginate. Chitosan, a product of deacetylation of chitinn, is a positively charged biopolymer and the charge density is directly related to the degree of deacetylation (Fig. 4.2) (Bhatia et al., 2005). Chitosan can be complexed with alginate and partly replace Ca^{+2}, to make a new layer on the Ca-alginate capsules.

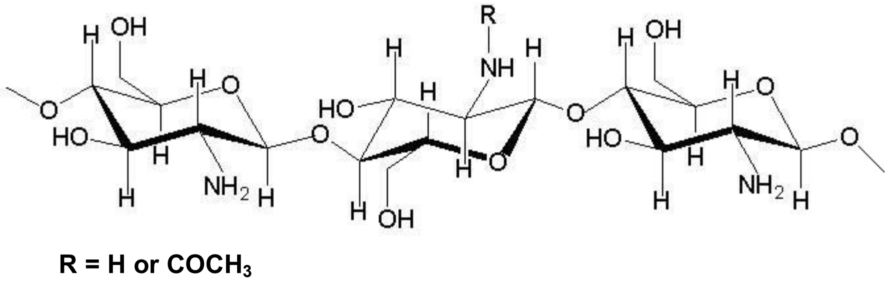


Figure 4.2. Structure of chitosan, NH_2 is turned to NH_3^+, depending on the pH of the solution.

Chitosan with a low molecular weight is preferred, due to its deeper penetration into the Ca-alginate matrix. The presence of calcium ion to some extent facilitates the linkage of alginate to chitosan with a mechanism not yet well understood (Gaserod et al., 1998). Amino groups make chitosan a cationic polyelectrolyte ($pK_a \approx 6.5$) and therefore, it is soluble only in aqueous acidic media at pH lower than 6.5 where amino groups are positively charged (Krajewska, 2004). Ammonium ions (NH_3^+) can link to the carboxyl groups (COO^-) on the alginate backbone and result in a stronger network. This linkage is not sequestered by phosphate or citrate ions. For coating of Ca-alginate capsules, a solution consisting of 0.2% chitosan and 0.3 M $CaCl_2$ dissolved in 0.04 M acetate buffer pH 5 was first prepared. The capsules were then immersed in chitosan solution with a volume ratio about 1:5 and placed in a shaker bath at 30°C and 140 rpm for 24 h. Next, the coated and uncoated capsules were added into a citrate solution in order to dissolve Ca-alginate membrane. The alginate-chitosan membrane is shown in Fig. 4.3a.

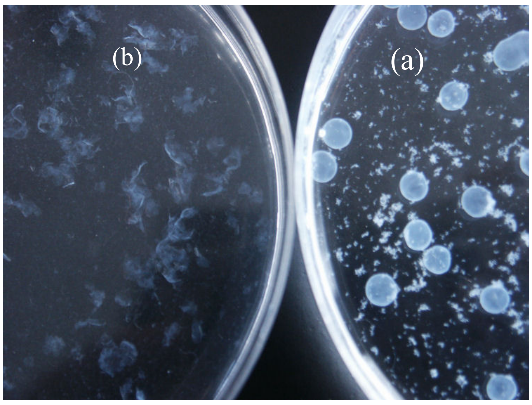


Fig. 4.3. Treatment of Ca-alginate capsules covered with (a) and without (b) an extra chitosan layer.

In parallel, attempts were made to reduce the size of capsules because smaller beads offer many advantages such as higher rate of diffusion for nutrients and products, improved mechanical stability and better cell function (Canaple et al., 2002; Sakai et al., 2006; Sakai et al., 2005). The size of a liquid drop extruded through a needle is controlled by the gravity force, drag force and surface tension. The size of liquid drop can be reduced with a superposed air stream, which flows in the concentric annulus and increases the drag on a liquid drop formed at the end of the inner needle. Ca-alginate capsules with mean diameter of 1.8 (±0.2) mm were made using a coaxial air-flow method (Fig. 4.4).

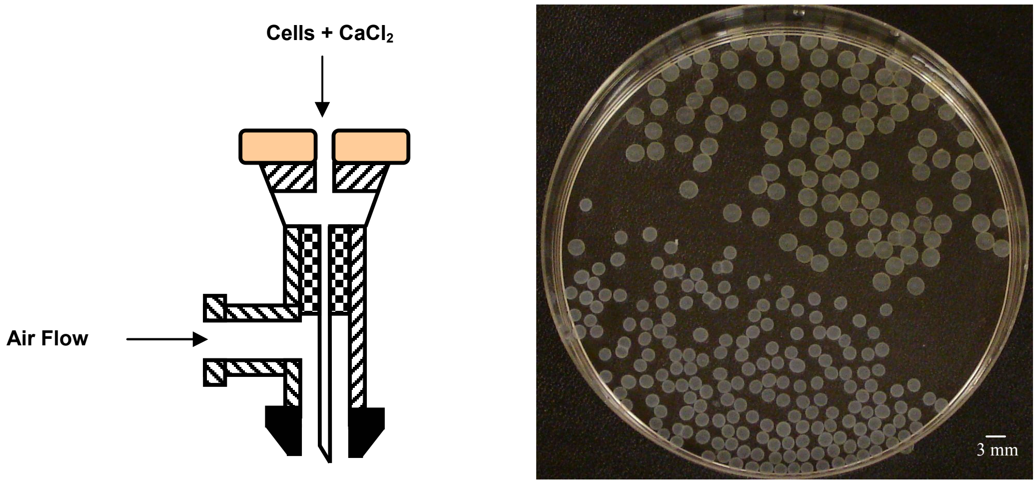


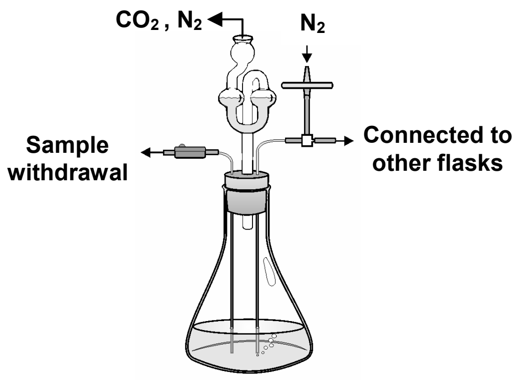
Fig. 4.4. Coaxial air-driven extruder and the capsules produced at different sizes.

4.5. Cultivation conditions

4.5.1. Batch cultivation

The anaerobic experiments were carried out in a set of 300 ml conical flasks placed in a shaker bath at 30°C. The liquid volume was 100 ml with ca. 30 ml of cell-seeded capsules, and the shaker speed was 130 rpm. Each flask was equipped with a thick silicone rubber stopper, two stainless steel capillaries, and a glass tube with a loop trap as previously described (Taherzadeh et al., 1997a). The capillaries were used for drawing samples with a syringe and injecting nitrogen gas (Fig. 4.5).

Fig. 4.5. Conical flask for anaerobic batch cultivation.



To provide anaerobic conditions, nitrogen gas (with less than 5 ppm O_2) was sparged into the flasks. Sterile glycerol was used in the loop-traps to prevent oxygen back-diffusion to the medium, while permitting nitrogen and produced CO_2 to leave the flasks.

4.5.2 Continuous cultivation

Continuous cultivation was carried out in a 2.5-liter Biostat A bioreactor (B. Braun Biotech, Germany). Similar to batch cultivation, temperature and pH were set at 30°C and 5.0 respectively during the cultivation. Control of operational parameters such as stirring rate, temperature, pH, culture volume, antifoam addition, etc. was accomplished by an integrated controller, microDCU 300 (B. Braun Biotech, Germany). A peristaltic pump (Watson-Marlow Alitea AB, Sweden) was used to feed the substrate into the bioreactor. The injection of gas flow (either air or N_2 for aerobic and anaerobic cultivation) was regulated by a Hi-Tech mass flow controller (Ruurlo, The Netherlands). A gas analyzer (model 1311, Innova, Denmark) was integrated to the system for on-line measurement of exhaust gas compositions (CO_2 and O_2). A base dosing pump triggered by the controller was utilized to control the pH. A program on LabVIEW platform (National Instrument) developed for Microsoft Windows was used for on-line measurements, instruments control, and data acquisition.

4.6. Analyses

4.6.1. HPLC

The concentration of chemical components in the hydrolyzates as well as components and metabolites in the cultivation medium were quantified by a high-performance liquid chromatography (HPLC) system. The Alliance HPLC System used in the current study, includes separations module (Waters 2695, Milford, MA), which offers integrated solvent and sample management and consists of a mobile phase reciprocating pump and an auto-sampler; a column oven; and three different detectors, namely, for refractive index (RI, Waters, 2414), UV absorbance (Waters, 2487) and evaporative light scattering (ELS, Waters, 2420). Data acquisition software, Empower 2, was used as interface to perform analysis, retrieve data and control the entire system.

Two different columns from Bio-Rad (Hercules, CA), Aminex HPX-87H and Aminex HPX-87P, packed with a polystyrene divinylbenzene resin with respective ionic form of hydrogen and lead, were utilized in the current work. Compounds are separated on Aminex HPLC columns using the ion-moderated partition chromatography (IMP) technique, which separates molecules based on a number of different chemical characteristics. Aminex HPX-87H column at 60°C with 5 mM H_2SO_4 as eluent was used for analysis of organic acids such as acetic acid, succinic acid, galactrunic acid, furoic acid and other compounds including glycerol, glucose, ethanol, furfuryl alcohol, furfural and HMF. Mixture of the sugars including glucose, xylose, galactose, arabinose and mannose was analyzed by ion- exchange Aminex HPX-87P column at 85°C where ultra-pure water was used as eluent. Furfural, HMF, furfuryl alcohol, and furoic acid concentrations were determined from the UV chromatograms at 210 nm, whereas concentrations of the rest components were determined from the RI chromatograms. The ELS detector was also used to measure concentration of various sugars. Volatile organic compounds such as ethanol, furfural and acetic acid in the analyzing sample are evaporated in this detector. Therefore, interference between volatile and non-volatile compounds is avoided, which could lead to better and more dependable results.

4.6.2. Gas Chromatography (GC)

In order to determine the concentration of limonene, hydrolyzate sample (100 ml) was added to n-heptane (40 ml, 99% purity) and centrifuged at 3500×g for 30 min to extract the oil. The resulting supernatant was then analyzed by gas chromatography coupled with a mass spectrometer (GC-MS). The analysis was performed on a HP G 1800c GCD series II (Hewlett Packard, Agilent, Palo Alto, CA) equipped with a capillary column HP-5 (30m × 0.25 mm i.d. ; stationary phase made of a film of diphenyldimethyl- polysiloxane 5%, with 0.25 μm thickness). The initial temperature was 50°C and increased up to 250°C with temperature ramp of 15°C/min and maintained at this temperature for 3 min. Helium was used as the carrier gas (Fernandez et al., 2005; Simi et al., 2004).

4.6.3. Analysis of intracellular components of yeast cells

The changes in the morphology, growth pattern and physiology of immobilized cells have been previously reported (Doran and Bailey, 1986; Melzoch et al., 1994). In the current work, the physiological responses of the encapsulated cell to the altered environmental conditions were investigated with respect to their internal composition changes over long-term application (paper IV). Therefore, protein, total carbohydrates, RNA, and two important carbohydrate reserves – that is, glycogen and trehalose content of yeast cells – were analyzed. Total protein content of cells was determined by addition of 3 ml 1M NaOH to samples previously washed and stored in the freezer. The pellet was then suspended and boiled for 10 min. After cooling on ice, the protein content was measured by the Biuret method (Verduyn et al., 1990b). Bovine serum albumin (Sigma, A-7908) was used as a standard.

RNA content of cells was estimated based on a method proposed by Benthin et al. (1991), where RNA was extracted in perchloric acid and degraded by alkali. In this method, the cells were washed three times with 0.7 M $HClO_4$ and digested with 0.3 M KOH for 1 h at 37°C with occasional mixing. The supernatant was cooled and neutralized with 3M $HClO_4$. After centrifugation, the supernatant was again collected and the precipitate was washed twice with 0.5 M $HClO_4$. Extracts from different steps were collected and centrifuged once again and used for absorbance measurement at 260 nm. The RNA concentration was determined using the average nucleotide data (M_W = 340 g/mol, ε = 10,800 $M^{-1}cm^{-1}$) as:

$$mgRNA = \frac{vM_W}{\varepsilon} \times A_{260} \tag{4.2}$$

where v is the volume of the extracts in ml.

Trehalose and glycogen content of yeast cells were determined according to Parrou and Francois (1997). In brief, 4-10 mg dry weight of yeast cells were suspended in 0.25 ml of

0.25 M Na_2CO_3 using screw-top Eppendorf tubes and incubated at 95°C for 4 h. The pH of the mixture was adjusted to 5.2 by addition of a buffer solution including 0.15 ml 1 M acetic acid and 0.6 ml 0.2 M Na-acetate. The suspension was then divided into two equal volumes, where the first part was incubated with trehalase (approximately 0.05 U/ml) overnight at 37°C and another part with *Aspergillus niger* amyloglucosidase (1.2 U/ml) at 57°C with continuous agitation. The suspension was then centrifuged and the glucose liberated was determined by enzymatic kit (Boehringer, Cat. No. 10716 251 035). The results were expressed as the percentage of glucose equivalent to glycogen or trehalose per cell dry weight (% w/w).

The colorimetric method for the determination of total carbohydrate by the phenol-sulfuric acid reagent was applied according to Herbert et al. (1971). The absorbance of liberated sugars was measured at 488 nm and glucose (2 g/l) was used as standard solution.

5. FERMENTATION OF UNDETOXIFIED HYDROLYZATES

Formation of many toxic compounds during dilute-acid hydrolysis of cellulosic materials makes the resultant hydrolyzate a severely harsh medium for *S. cerevisiae* in the fermentation step, which usually leads to very low ethanol productivity or even complete failure of cultivation. Different strategies have been suggested to overcome this problem, including biological, physical or chemical detoxification, using high cell mass density for instance by cell recirculation or cell immobilization and development of improved inhibitor-tolerant microorganisms by screening or genetic manipulation (Almeida et al., 2007; Brandberg et al., 2005; Chung and Lee, 1985; Nilsson et al., 2001; Palmqvist and Hahn-Hägerdal, 2000; Taherzadeh et al., 2001).

Some inhibitors present in the hydrolyzate, such as furans and phenolics, can be metabolized by yeast and converted to less inhibitory compounds (Boyer et al., 1992; Chung and Lee, 1985; Palmqvist and Hahn-Hägerdal, 2000). This capability is known as *in situ* detoxification capacity of yeast cells. The *in situ* detoxification capacity of yeast has been applied to develop a fed-batch process for cultivation of severely inhibiting hydrolyzates (Nilsson et al., 2001; Taherzadeh et al., 1999b). Although this method was successful, it showed a drastic decrease in cell viability as soon as the feed rate or toxicity of the hydrolyzate was increased (Nilsson et al., 2001; Nilsson et al., 2002). This fact suggests that a successful fermentation process with hydrolyzate can proceed if the feed rate of inhibitory compounds is lower than the *in vivo* detoxification capacity of yeast cells. From the results of paper II, it was postulated that encapsulation improves the *in situ* capacity of cells for detoxification, as fermentation of inhibitory synthetic medium and undetoxified hydrolyzates was far more successful than the free cells. This could be attributed to high cell concentration and specific growth pattern of encapsulated cells.

A continuous mode of operation has been of interest to cultivate the hydrolyzates due to many advantages including higher conversion and faster fermentation rates, reduced product losses and higher volumetric ethanol productivity (Verbelen et al., 2006). More importantly, the rate of inhibitors addition to the bioreactor can be controlled at the levels beyond the capacity of cells for *in situ* detoxification when toxic medium such as hydrolyzates are to be fermented.

5.1. Difficulties in fermentation with free cells

5.1.1. Batch cultivation

The effect of furfural in lag phase prolongation or even failure of the cultivation by *S. cerevisiae* is already known. Sanchez and Bautista (1988) reported complete inhibition of fermentation with *S. cerevisiae* at furfural concentration of 2 g/l. In another report, furfural at a concentration of 4 g/l inhibited the growth of *S. cerevisiae* and alcohol production by 80% and 97%, respectively (Banerjee et al., 1981). Fermentation rate of glucose medium in the presence of 3 g/l was only 33% of that in furfural-free medium (Azhar et al., 1981). In free-cell cultivation of yeast *S. cerevisiae* CBS 8066 in the synthetic defined medium containing 5 g/l furfural, no growth, sugar consumption and ethanol production occurred for at least 24 h. Similar observations were made with cultivation of freely suspended yeast cells in two different undetoxified wood hydrolyzate media, stages 1 and 2 (paper II).

Successful cultivation of orange peel hydrolyzate by free cells depends strongly on the concentration of released peel oil in the medium. The effect of limonene in defined media on viability of freely suspended yeast cells (CBS 8066) was investigated using the colony-forming units method (CFU). The fraction of viable cells in defined media containing 0.5, 1 and 1.5% v/v of limonene decreased to 51, 36 and 25% of the initial values, respectively, within 1 h cultivation and after 3-4 h cultivation, the number of viable cells for all media containing limonene was practically zero (Fig. 5.1). Anaerobic batch cultivations of freely suspended cells in the same media failed and the cells were not able to assimilate glucose in any of these media containing limonene at different concentrations (paper V).

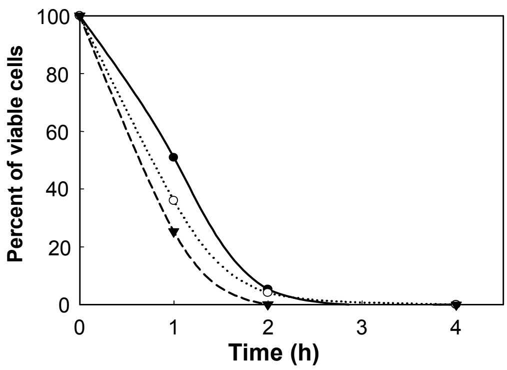


Fig. 5.1. Effect of 0.5% (●), 1% (○), 1.5% (▼) limonene on viability of *S. cerevisiae* CBS 8066 in batch cultivation with suspended cells (paper V).

Enzymatically hydrolyzed peels were supplemented with nutrients (except carbon source) and anaerobically cultivated by freely suspended cells. The limonene content of hydrolyzate was measured as 0.52% v/v. As expected, the suspended cells were not able to ferment the peel hydrolyzate in 24 h and no ethanol was produced (paper V).

5.1.2. Continuous cultivation

In free-cell cultivation in an ideal chemostat or continuous-flow stirred tank reactor (CFSTR) at steady-state conditions, the cells are removed at a rate equal to their growth rate, which is in turn equal to the dilution rate (Shuler and Kargi, 2002). When the rate of cell removal from the reactor exceeds the growth rate of cells, wash-out occurs. Therefore, in the case of reduced growth rate of cells, e.g. when toxic hydrolyzates are used, the continuous cultivation is feasible only at very low dilution rates. Fermentability of the hydrolyzate was investigated using freely suspended *S. cerevisiae* in continuous cultivation with an initial cell concentration of 4.81 g/l. Even though the steady-state condition was accomplished at 0.1 h^{-1} and 90% of hexose sugars were converted, the cell viability measured by CFU decreased to 40% and 25% after 2.5 and 5 retention times, respectively (Table 5.1). The ethanol yield was 0.34 g/g and right after switching to 0.2 h^{-1} dilution rate, the carbon dioxide evolution rate decreased drastically and approached zero, fermentation ceased and all cells were washed out from the bioreactor (paper III).

Table 5.1. The most important results of anaerobic continuous cultivation of dilute-acid hydrolyzate at steady-state conditions by "freely suspended" *S. cerevisiae* (paper III).

D (h^{-1})	Conversion (%)			Y_{SE} (g/g)	Y_{SGly} (g/g)	r_E (g/l·h)	q_E (g/g·h)	Viability (%)
	Hexose	Furfural	HMF					
0.1	90 ±2	100	48 ±3	0.34±0.03	0.036	0.857	0.53	25 ±2
0.2	Wash-out							

Note: Calculations of ethanol and glycerol yields were based on the sum of the consumed glucose, mannose and galactose.

5.2. Cultivation with encapsulated cells

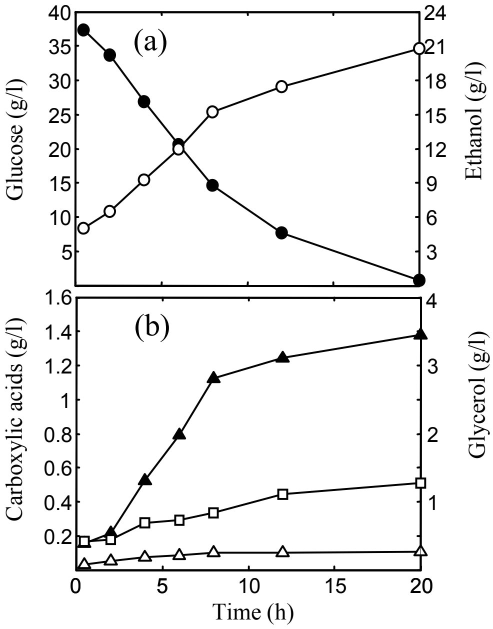
5.2.1. Batch cultivation

In the fist stage of using high cell concentration to confront inhibition of fermentation of hydrolyzates, the yeast cells were encapsulated and cultivated either in defined synthetic medium containing high concentration of inhibitor (5 g/l furfural) or in highly toxic hydrolyzates medium. In the current work, encapsulated *S. cerevisiae* was applied to ferment a synthetic medium containing 5 g/l furfural. The encapsulated cells were able to successfully tolerate furfural and take up the sugar with almost no lag phase (Fig. 5.2).

The results indicate that the rate of glucose consumption and ethanol production decreased due to the presence of furfural and the cultivation was completed in longer time compared to medium without furfural. Although the ethanol productivity was clearly lower than in the corresponding experiments without furfural in all 5 batches, it was still higher than the corresponding value in the free cells without furfural (Paper II).

While the ethanol yield was less affected, the yield of glycerol was highly affected by the addition of furfural to the media and decreased to less than half of the value for the cultivation in furfural-free media. The acetate yield in the first batch was 0.034 g/g and increased linearly during sequential batches and reached 0.068 g/g in the fifth batch. Hence, the acetate yield obtained was significantly higher than the same value in furfural-free cultivation. The presence of furfural had no significant effect on the yield of succinic acid from the encapsulated cells (paper II).

Although the cultivations lasted for more than 20 h, all furfural was converted by the encapsulated yeast in less than 8 h (cf. Fig. 5.2 a,c). The majority of furfural was reduced to furfuryl alcohol and to a minor amount of furoic acid by the encapsulated cells (Fig. 5.2 c). Reduction of furfural to furfuryl alcohol is most likely catalyzed by the action of NAD(P)H-dependent alcohol dehydrogenase (ADH) enzyme, resulting in a reduced intracellular NAD(P)H level (Almeida et al., 2007; Modig et al., 2002; Palmqvist et al., 1999; Sarvari Horvath et al., 2001). The decrease in glycerol yield and increase in acetic acid yield in the presence of furfural can be well explained from the cellular redox balance. Under anaerobic conditions, glycerol is formed to re-oxidize the surplus cytosolic NADH, but formation of acetic acid from glucose involves production of NADH and therefore, demanded NAD(P)H for reduction of furfural to furfuryl alcohol is provided through less production of glycerol and further production of acetic acid (Sarvari Horvath et al., 2003).



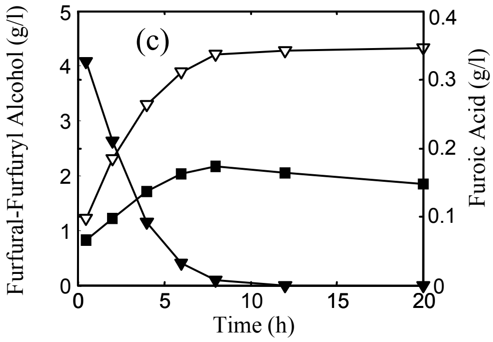


Fig. 5.2. Anaerobic batch cultivation of encapsulated *S. cerevisiae* in synthetic media containing 5 g/l furfural. The profiles are **(a)** glucose (●), ethanol (○); **(b)** glycerol (□), acetic acid (▲), succinic acid (△); **(c)** Furfural (▼), Furfuryl alcohol (▽) and Furoic acid (■) concentrations (paper II).

Production of ethanol from dilute-acid hydrolyzate by the encapsulated cells was quite successful for the first batch cultivation. There was no lag phase and the ethanol productivity was even higher than the cultivation of the synthetic medium by the free cells (paper II). However, a stepwise decrease in the yield of ethanol and its productivity from sequential cultivation of the hydrolyzates by the encapsulated yeast was observed. This could be due to gradual deactivation of cells exposed to highly toxic hydrolyzates over time and consequent reduction of the cells' viability (Nilsson et al., 2002).

Since the major difficulty in cultivation of orange peel hydrolyzate is the presence of peel oil, application of a selective membrane could lead to elimination of the problem. Capsules' membrane composed of Ca-alginate with hydrophilic nature is not theoretically permeable for components with hydrophobic nature like limonene (paper V). The membrane is thus a selective barrier that protects the yeast cells by allowing inward/outward permeation of nutrients and products while preventing oil from passing through the membrane and being in direct contact with the yeast cells. As a result, direct exposure of cells to the peel oil is avoided. The results of anaerobic batch cultivation of encapsulated *S. cerevisiae* in the same fashion as free-cell cultivation in synthetic medium containing high concentration of limonene confirmed this idea, although a minor amount of limonene was detected in the liquid core of the capsules. Cultivation of encapsulated cells was successful and glucose was assimilated even at very high limonene concentration of 1.5% v/v (Table 5.2). However, cultivation in the limonene-free medium was faster and completed in 5 h, while the cultivations in the presence of 0.5 to 1.5% v/v limonene ended in 7 h. Nevertheless, increasing the limonene concentration in this range had practically no effect on the fermentation time (paper V).

Table 5.2. Key rates and yields in anaerobic batch cultivation of suspended cells in limonene-free medium and encapsulated cells in media with various concentrations of limonene and peel hydrolyzates (paper V).

Parameter	**Encapsulated cells in media with different limonene concentration (% v/v)**				**Peel hydrolyzate**
	0	**0.5**	**1**	**1.5**	
$-r_S$ (g/l·h)	8.56	5.96	5.38	5.58	5.00
r_E (g/l·h)	3.69	2.57	2.28	2.33	2.18
Y_{SE} (g/g)	0.43	0.43	0.42	0.42	0.44
Y_{SGly} (g/g)	0.062	0.050	0.048	0.047	0.050
Y_{SAce} (g/g)	0.017	0.016	0.016	0.015	0.017

Ethanol production and glucose uptake rate were also affected by presence of limonene. Ethanol productivity in the media containing zero, 0.5, 1, and 1.5% v/v limonene was 3.69, 2.57, 2.28 and 2.33 g/l·h, respectively which shows a clear difference between the presence and absence of limonene in the culture. However, the effect of increasing limonene concentration by three-fold from 0.5 to 1.5% v/v on the ethanol productivity might be in the range of experimental variation. Ethanol productivity of the encapsulated cells in presence of limonene was still higher than the corresponding value with the suspended cells in limonene-free medium (cf. paper V). No significant effect of limonene at different concentration on the yields of ethanol, acetic and succinic acids was observed. Yield of glycerol, on the other hand, was the only variable influenced by the presence of limonene. Yield of ethanol remained constant at 0.425 (±0.005) g/g while glycerol yield was slightly decreased from 0.062 to 0.050 g/g with 0.5% limonene in the medium. Further increase in limonene concentration, however, had a minor effect on reduction of glycerol yield (Table 5.2).

Encapsulated *S. cerevisiae* successfully converted the fermentable sugars in peel hydrolyzate to ethanol and other metabolites (Fig. 5.3). Among the sugars available in peel hydrolyzate, only glucose and fructose could be assimilated by applied yeast strain and fermentation was completed within 7 h.

Consumption of fructose was delayed by presence of glucose and the yeast started to take up fructose after the concentration of glucose approached below 5 g/l. Ethanol yield based on total sugar consumption was 0.44(±0.01) g/g, and yields of glycerol, acetic and succinic acids were similar to the corresponding values of 0.5% v/v limonene in synthetic medium (Table 5.2).

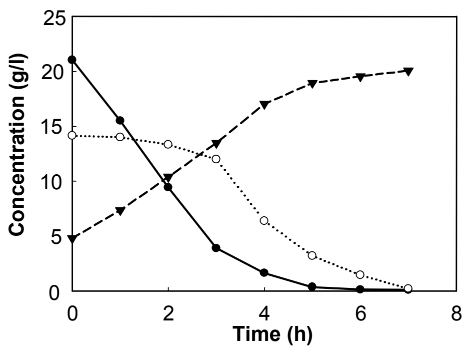


Fig. 5.3. Profiles of glucose (●), fructose (○) and ethanol (▼) in cultivation of orange peel hydrolyzate by encapsulated *S. cerevisiae* (paper V).

5.2.2. Continuous cultivation

The dependence of dilution rate on the growth rate of cells is eliminated by using immobilized cells due to cells retaining in the reactor and consequently, dilution rate higher than wash-out is achievable (Nigam, 2000). The applied yeast in a defined medium has a maximum specific growth rate (μ_{max}) of about 0.45 h^{-1} under anaerobic conditions (Taherzadeh et al., 1997b) and hence, continuous cultivation with free cells at dilutions rate more than 0.45 h^{-1} leads to wash-out. In this work, encapsulated cells were anaerobically cultivated in synthetic glucose medium and fermentation was performed at dilution rates up to 1.0 h^{-1} (paper III). The encapsulated yeast cells were able to consume glucose at all tested dilution rates, but increasing the dilution rates more than 0.4 h^{-1} resulted in a gradual increase of residual glucose in the outflow of the bioreactor. While almost all glucose was assimilated up to dilution rate 0.4 h^{-1}, about 37% the glucose remained unconsumed at dilution rate 1.0 h^{-1} (Table 5.3). The volumetric ethanol productivity was monotonically increased from 1.64 to 6.50 g/l·h from the lowest to highest dilution rates, while the specific ethanol productivity was firstly increased to 0.29 g/g·h up to 0.4 h^{-1} dilution rate and then slightly declined to 0.19 g/g·h at the highest rate (Table 5.3).

Table 5.3. Summary of the most important results of anaerobic continuous cultivation of encapsulated *S. cerevisiae* on glucose at steady-state conditions (paper III).

D (h^{-1})	Glucose conversion (%)	Ethanol			Other metabolites		
		Y_{SE} (g/g)	r_E (g/l·h)	q_E (g/g·h)	Y_{SGly}(g/g)	Y_{SAce}(g/g)	Y_{SSuc}(g/g)
0.2	100	0.40 ± 0.03	1.64	0.26	0.097	0.005	0.003
0.4	99.2 ± 0.5	0.44 ± 0.01	3.62	0.29	0.080	0.007	0.002
0.5	93.1 ± 0.4	0.44 ± 0.01	4.89	0.22	0.078	0.009	0.003
0.8	72.3 ± 1.5	0.43 ± 0.01	5.99	0.20	0.077	0.007	0.003
1.0	63.0 ± 2.0	0.43 ± 0.02	6.50	0.19	0.076	0.007	0.003

Continuous cultivation of the hydrolyzate by encapsulated *S. cerevisiae* was carried out by 250 ml capsules containing 4.02 g biomass in a total volume of 1 l medium. The experiments were initiated at dilution rate 0.1 h^{-1}, followed by a stepwise increase to 0.5 h^{-1}, where the feed rates were kept constant for at least 5 retention times to reach steady-state conditions. The cells assimilated glucose, mannose and galactose, while xylose could not be taken up by the yeast. The yeast was able to ferment hexoses to ethanol at all dilution rates, which is considered a success in cultivation without any prior detoxification (Table 5.4). While more than 95% of fermentable sugars were assimilated to ethanol at dilution rate 0.1 h^{-1}, the cells left 29% of initial glucose at rate 0.5 h^{-1}. The average ethanol yield was 0.44 (±0.01) g/g, and the volumetric ethanol productivity (r_E) proportionally increased with dilution rate, while the specific productivity (q_E) was almost constant at 0.16 g/g·h regardless of the dilution rate (Table 5.4).

The glycerol produced at different dilution rates had the same trend as the cultivation in synthetic medium, i.e. a gradual decrease with increasing dilution rate. However, the absolute values of yields were lower than those in the synthetic medium, and of the same order of free-cell cultivation in the hydrolyzate (cf. Tables 5.1 and 5.4). Furfural was totally converted at low dilution rates 0.1 and 0.2 h^{-1}, while the cells could not convert it totally at higher rates. On the other hand, the yeast cells were not able to convert HMF by more than 71%, which occurred at low dilution rates (Table 5.4). In contrast to free-cell cultivation in hydrolyzate, the fraction of viable cells estimated by colony-forming units (CFU) showed an increasing trend with dilution rates when encapsulated cells were used. More than 75% of cells were viable at tested dilution rates, and cells' viability was not seriously influenced at higher dilution rates (paper III).

Table 5.4. Summary of the key results of anaerobic continuous cultivation of dilute-acid hydrolyzate by encapsulated *S. cerevisiae* at steady-state conditions (paper III).

D (h^{-1})	Conversion (%)				Y_{SE} (g/g)	Y_{SGly} (g/g)	r_E (g/l·h)	q_E (g/g·h)
	Glucose	Mannose	Furfural	HMF				
0.1	95 ± 1	98 ± 1	100	71 ± 3	0.44 ± 0.01	0.052	1.146	0.17
0.2	84 ± 2	87 ± 3	100	57 ± 8	0.45 ± 0.01	0.044	2.053	0.16
0.3	79 ± 5	79 ± 7	92 ± 5	71 ± 9	0.43 ± 0.02	0.041	2.717	0.14
0.4	72 ± 6	90 ± 5	75 ± 4	52 ± 7	0.45 ± 0.02	0.041	3.545	0.15
0.5	71 ± 5	79 ± 4	69 ± 6	48 ± 5	0.45 ± 0.03	0.039	4.206	0.16

6. PHYSIOLOGICAL AND MORPHOLOGICAL STUDIES

6.1. Diffusion through the capsules' membrane

It was of interest during the current work to find out if the capsules' membrane shows any effect on selective permeation of different components, and therefore, higher tolerance of encapsulated cells can be at least partially explained by the protective effect of the membrane. The diffusion rates of four components, namely glucose, furfural, HMF and acetic acid, through the membranes of cell-free capsules were determined. However, the results indicated that the membrane had no selectivity in penetration of sugars or inhibitors during the cultivation. All the components needed 20 and 40 minutes to reach 90 and 99%, respectively, of final concentration in equilibrium through the capsules' membrane (paper III). Therefore, permeation of the inhibitors and sugars through the membrane is hardly connected with improved tolerance and high capacity for *in situ* detoxification represented by encapsulated cells (Fig. 6.1a). Instead, this phenomenon could be related to the specific growth pattern of cells in the form of a packed community inside the capsules. It seems that this kind of direct physical contact of cells with each other and with the solid surface, and growing in a limited space, affects the cells' metabolism and modifies their growth pattern (Doran and Bailey, 1986). A similar experiment was carried out to determine the permeability of limonene through the membrane. Cell-free capsules were added into the medium with 1% v/v initial concentration of limonene. In the equilibrium state, the concentration of limonene in the liquid core of capsules was determined as 0.05% v/v (paper V).

Recalling from section 3.4, membrane fouling may occur during long-term application of encapsulated cells, resulting in a limited accessibility of cells in the inner layer of packed-cell community to the substrates. Thus, encapsulated cells may undergo different degrees of starvation depending on the position of the cells inside the capsules, which can affect their morphology and metabolism (Branyik et al., 2005). In order to characterize the cells' conditions with respect to the nutrients' availability, the volumetric mass transfer coefficient for diffusion of glucose through the cell-seeded capsules over consecutive batch cultivations was determined (paper IV). Encapsulated cells in a medium containing nutrients are assumed to constitute a two-phase system separated by the capsules' membrane. Mass transfer between two phases can be mathematically described as (Lewinska et al., 2002):

$$V_0 \frac{dC}{dt} = -kA(C - C_C) \qquad (6.1)$$

where C and C_C are the solute (e.g. glucose) concentrations in solution outside and inside the capsules, respectively; k and A are the mass transfer coefficient and total external surface area of capsules; V_0 is the volume of solution outside capsules, and t is time. Mass balance for solute in both phases gives:

$$\frac{d(V_0 C + V_C C_C)}{dt} = 0 \tag{6.2}$$

Assuming a constant solute concentration in the equilibrium state (C_{eq}) in both phases produces a direct relationship between C and C_C which can be incorporated in Eq. (6.1). This equation can be solved with the initial condition of $C_{t=0}=C_0$ to obtain:

$$C = C_{eq} + (C_0 - C_{eq})\exp\left(-\left[\frac{1}{V_0} + \frac{1}{V_C}\right]kAt\right) \tag{6.3}$$

In this model it is assumed that there is no concentration gradient in the capsule interior. With the ratio of V_C and V_0 applied in the experiments, which is 1:3, Eq. (6.3) is expressed as:

$$\frac{C}{C_0} = \frac{C_{eq}}{C_0} + (1 - \frac{C_{eq}}{C_0})\exp\left(-4\frac{Kt}{V_0}\right) \tag{6.4}$$

where $K= kA$ is the volumetric mass transfer coefficient.

The values of K in different batch cultivations were determined by fitting a theoretical curve from Eq. (6.3) to experimental data with a nonlinear regression program using MATLAB® in which the root-mean-square error between the experimental data and the model prediction was minimized (paper IV). The results are presented in Fig. 6.1 b and Table 6.1.

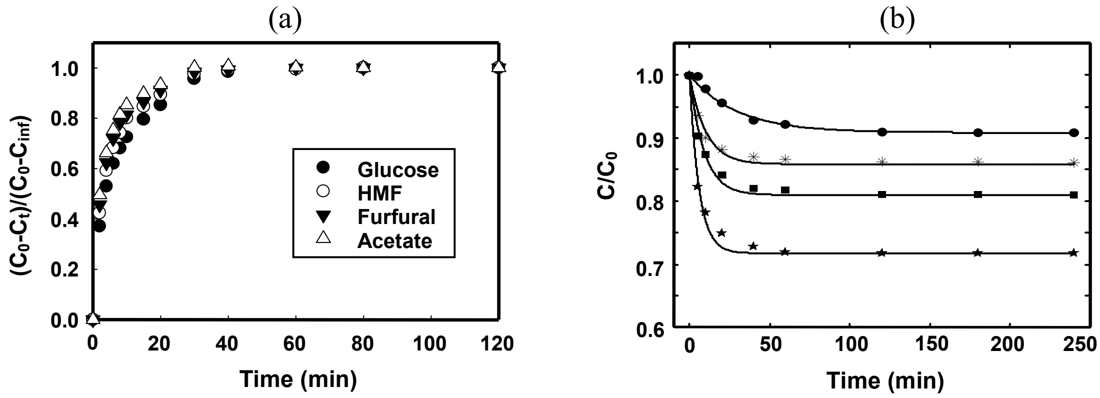


Fig. 6.1. (a) Profile of the solute diffusion through the capsules' membrane from bulk liquid (paper III), (b) Time course diffusion of glucose through the cell-free (★) and cell-seeded capsules' membrane after 5 (■), 10 (∗) and 20 (●) batch cultivations. The lines show the profile of diffusion according to the model using the estimated mass transfer coefficients (paper IV).

As the capsules containing cells were cultivated longer, the cell growth made the membrane less permeable for glucose, leading to a lower diffusion rate. The value of the volumetric mass transfer coefficient estimated for glucose penetrating through the membrane of cell-free capsules was 6.28 (cm^3/min) while the corresponding value for capsules containing cells showed a decreasing trend as the capsules were exposed to more batch cultivations. After the 20th batch cultivation, when the concentration of yeast cells inside the capsules was very high, the mass transfer coefficient had a value of 1.24 (cm^3/min) which is only 20% of its initial value for cell-free capsules (Table 6.1).

Table 6.1. Calculated volumetric mass transfer coefficient (K) of glucose into empty capsules and the capsules collected from different batches of the sequential cultivations with encapsulated cells (paper IV).

Samples	**K (cm^3/min)**
Cell-free capsules	6.28
Capsules from the 5th batch	4.11
Capsules from the 10th batch	3.70
Capsules from the 20th batch	1.24

6.2. Growth rate and morphological study

The growth rate of immobilized cell system (mostly prepared by other methods than encapsulation) compared to free-cell culture has been the subject of many studies where discordant results have been reported indicating an increased, static or decreased value. Internal mass transfer limitation of nutrients constitutes the most evident hypothesis to explain the lower immobilized cell growth rate, while a higher rate is attributed to the protective effect of immobilization carrier. It is, however, noteworthy that comparison of the results of immobilized cell systems prepared by different techniques, having different matrices and variable system configurations with each other and/or with the free-cell system, is rather difficult. This fact at least partly explains the contradictory results achieved from immobilized cell systems (Branyik et al., 2005; Junter et al., 2002; Norton and D'Amore, 1994).

Morphological study of encapsulated yeast cells released from the membrane was performed using microscopic inspection (Fig. 6.2). The average fraction of budding yeast estimated on a Bürker counting chamber was gradually reduced over the sequential batch cultivations. Many cells in the later cultivation batch exist in the form of single and non-budding cells (paper IV).

In the current work, the specific growth rate of the encapsulated cells was low compared to the freely suspended cells. This fact is in agreement with the reduced fraction of budding yeast over the cultivation sequences. Both glucose limitation due to the lower rate of mass transfer (Fig. 6.1b) and cell attachment to the membrane and/or other cells might be responsible for inhibition of budding in encapsulated cells in the inner layer of the cell community (Doran and Bailey, 1986).

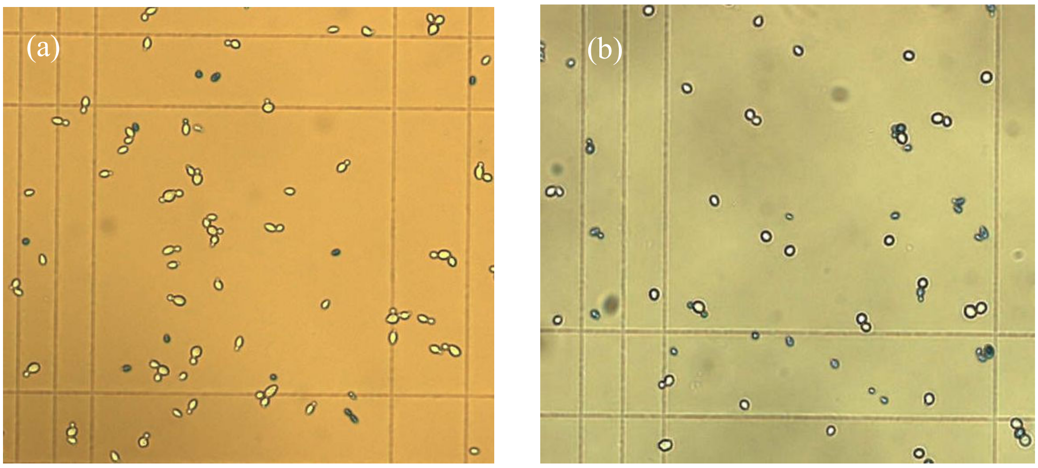


Fig. 6.2. Photograph of encapsulated yeast cells, released from the membrane and inspected with light microscopy (magnification ×400), (a) after batch 1, (b) after batch 20.

6.3. Physiological study of encapsulated yeast

Altered microbial physiology in immobilized cells has been shown by certain authors, and it is known that immobilized cells have properties different from those of free cells (Doran and Bailey, 1986; Junter et al., 2002; Shen et al., 2003). The cause of these changes is not yet fully elucidated, and they are often attributed to modification of microenvironment around the immobilized cells. Two important features which are usually absent in the case of free-cell cultivation are considered to be responsible for these modifications: cell attachment on solid surfaces, and mass transfer limitations through the immobilization matrix as discussed earlier (Norton and D'Amore, 1994; Shen et al., 2003).

Through this research, the physiological responses of the encapsulated cell to the altered environmental conditions, with respect to their internal composition changes over long-term application, were investigated (paper IV). To get a better understanding of cells' behavior during encapsulation, the total carbohydrates, RNA, protein, and two important carbohydrate reserves, i.e. glycogen and trehalose content of yeast cells, were analyzed. The cellular RNA content is an important parameter in studying yeast physiology because it is highly related to the specific growth rate of yeast. The decrease of total RNA content of yeast cells due to growth impairment in both linear and non-linear fashion has been reported (Sebastian et al., 1973; Waldron and Lacroute, 1975). The biomass samples obtained after mechanical rupture of capsule membranes were utilized for determination of cellular compositions. The RNA content from samples retrieved was 9.03% of the biomass dry weight after the first batch. It steadily decreased over the repeated cultivations and reached around 5.5% by 20 batch sequences (Fig. 6.3 a). This means that the average amount of total RNA as a percentage of dry weight decreased by 39% over 20 consecutive batch cultivations (paper IV).

Protein together with RNA constitute are termed protein-synthesizing system (PSS), and thus it is reasonable to expect that a decrease in RNA content will lead to a decrease in protein content and, accordingly, that a higher growth rate results in a higher amount of PSS (Schulze, 1995). The protein content of the cells from sequential batches followed the same trend as RNA content (Fig. 6.3 b). Protein content was approximately 46% w/w after the first cultivation of the encapsulated cells, while it continuously decreased to 35% w/w in the last cultivation, showing a 24% decrease over these 20 cultivation batches (paper IV).

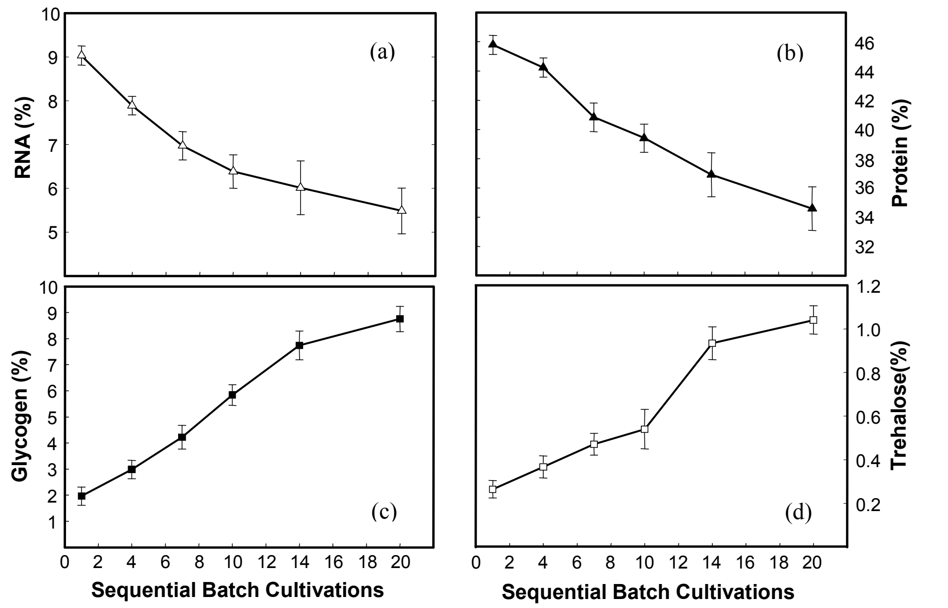


Fig. 6.3. Profile of (a) total RNA, (b) total protein, (c) glycogen and (d) trehalose content (% w/w) of encapsulated yeast samples at different sequential batch cultivations (paper IV).
Note: The results of glycogen and trehalose are expressed as the percentage of glucose equivalent per cell dry weight (% w/w).

Glycogen and trehalose are two reserved carbohydrates accumulated under slow growth of microbial cells due to nutrient limitation, and accurate estimation of their concentration is essential for detailed understanding of many aspects of yeast physiology. Trehalose is also accumulated in cells exposed to environmental stress (Aranda et al., 2004). Encapsulated yeast cells accumulated both the aforementioned carbohydrates as they were growing over repeated batches. The glycogen and trehalose contents at the end of the first batch were 1.96% and 0.26% of the dry weight of biomass. Both carbohydrates' content increased gradually after each batch cultivation. The corresponding values in the last cultivation for glycogen and trehalose were respectively

4.5 and 4 times higher than those from the first batch. The sum of glycogen and trehalose accounted for 2.22, 6.37 and 9.80% of the total cell mass after batches 1, 10 and 20, respectively (Fig.s 6.3 c,d) (paper IV).

Total carbohydrate measurements also showed an elevated content over sequential batches, and followed trends similar to those of the two stored carbohydrates. From the first to the 20th batch cultivations, the total carbohydrate content of yeast cells increased from 27.5 to almost 39%, indicating 11.5% rise in carbohydrate content of the yeast biomass (paper IV).

The growth rate proved to have little effect on structural carbohydrates in the cell wall, i.e. glucan and mannan. Consequently, increase in the average level of glycogen and trehalose could be attributed to the yeast cells inside the packed community, whose growth has been suppressed due to attachment and/or lack of nutrients, since the cells accumulate the stored carbohydrates under such conditions (Doran and Bailey, 1986). This unique growth pattern of cells with altered cell compositions and perhaps appearance of new proteins as reported by Perrot et al. (2000) could play a role in giving higher resistance to encapsulated yeast to survive and grow in highly toxic medium.

7. CONCLUDING REMARKS

Efficient fermentation of dilute-acid hydrolyzate has been the subject of large numbers of investigations where different strategies have been proposed to overcome the toxicity of the hydrolyzate. High cell concentration in the cultivation media can help in decreasing the fermentation time as well as increasing the tolerance of the cells against the inhibitors. On the other hand, aggregation is a natural defense mechanism for cells to face the harsh environmental conditions and to gain much better protection. Both concepts were applied in the current work to develop an encapsulated yeast cell system to improve *in situ* capacity of cells and their tolerance against inhibitors present in toxic medium such as wood hydrolyzates. The possibility of fermentation of toxic synthetic medium and undetoxified hydrolyzate in both batch and continuous cultivation shows the potential capacity of encapsulated yeast cells for fermentation of inhibitory media.

Both volumetric glucose consumption and ethanol production rates with encapsulated system are several times higher than those of free-cell cultivation. Cultivation with encapsulated cells results in lower glycerol and acetate yields and higher ethanol yield compared to free-cell system. Increased capacity of encapsulated cells for *in situ* detoxification along with high proportion of viable cells leads to continuous cultivation of undetoxified lignocellulosic hydrolyzate at dilution rates several times higher than those with free-cell cultivation. The volumetric ethanol productivity of encapsulated cells in hydrolyzate is comparable with that in synthetic medium without presence of any inhibitors. These advantageous features of encapsulated cells are most likely related to specific growth pattern of cells in the form of packed-cell community with physiological and morphological modification.

While capsules' membrane shows no selectivity with respect to components present in wood hydrolyzate, in orange peel hydrolyzate or limonene-contained medium it is highly selective, allowing successful fermentation of such medium in presence of 1.5% (v/v) limonene. The estimated concentration of limonene in the liquid core of capsules is only ca. 5% of that in bulk liquid outside the capsules.

8. FUTURE WORK

The present work is part of extensive research devoted to meeting challenges and requirements that human society currently encounters in sustainable development. Producing fuel from renewable resources is becoming an important issue for the whole world. Therefore, the work needs to be continued for further development of ethanol production from biomass. The following suggestions could be of interest for future studies:

- Although an encapsulated yeast cell seems to be a promising method among the immobilization techniques, some issues remain to be addressed. Development of a membrane with improved characteristics including biocompatibility, mechanical and chemical strength, availability, cost and long-term durability is among the most important challenges for cell encapsulation technology.

- Mass production of capsules is required in order to facilitate industrial application of encapsulated cell system. Production of uniform capsules with excellent repeatability and reproducibility within and between batches is also of importance.

- More detailed study of the physiological and metabolic modifications occurring for encapsulated cell using more advanced techniques is required to get a better understanding of cell behavior during long-term application.

- Other types of immobilization which share the most important features of encapsulation can also be considered. For instance, surface adsorption where the yeast cells are temporarily immobilized onto the solid surfaces seems to be more practical. Cell immobilization by self-flocculation could also be of interest due to economic and technological advantages. However, the resultant tolerance of cells and their performance in toxic medium must be further elucidated.

NOMENCLATURE

D	Dilution rate (h^{-1})
C_0	Initial concentrations of estimated components (mM)
C_t	Concentrations of estimated solutes in bulk liquid at time t (mM)
C_{inf}	Concentrations of estimated solutes in bulk liquid at equilibrium (mM)
CFU	Colony-forming units
Y_{SE}	Ethanol yield (g/g or mg/g)
Y_{SGly}	Glycerol yield (g/g or mg/g)
Y_{SAce}	Acetic acid yield (g/g or mg/g)
Y_{SSuc}	Succinic acid yield (g/g or mg/g)
Y_{SX}	Biomass yield (g/g or mg/g)
Y_{SC}	Carbon dioxide yield (g/g or mg/g)
q_E	Specific productivity of ethanol (g/g·h)
r_E	Volumetric productivity of ethanol (g/l·h)
μ	Specific growth rate (h^{-1})
k	Mass transfer coefficient (cm/min)
A	Total external surface area of capsules (cm^2)
K	Volumetric mass transfer coefficient (cm^3/min)
V_0	Volume of solution outside capsules (cm^3)
V_C	Volume of solution inside capsules (cm^3)
C	Solute concentration in solution outside capsules (g/l)
C_C	Solute concentration in solution inside capsules (g/l)
C_{eq}	Solute concentration in the equilibrium state (g/l)

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Paper I

OPTIMIZATION STUDY OF CITRUS WASTES SACCHARIFICATION BY DILUTE-ACID HYDROLYSIS

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The effects of time, acid concentration, temperature and solid concentration on dilute-acid hydrolysis of orange peels were investigated. A central composite rotatable experimental design (CCRD) was applied to study the individual effects of these hydrolysis factors and also their interdependence effects. The enzymatic hydrolysis of the peels by cellulase, β-glucosidase, and pectinase enzymes resulted in 72% dissolution of the peels, including 18.7% galacturonic acid and 53.3% of a total of glucose, fructose, galactose, and arabinose. Dilute-acid hydrolysis up to 210°C was not able to hydrolyze pectin to galacturonic acid. However, the sugar polymers were hydrolyzed at relatively low temperature. The optimum results were obtained at 116°C, 0.5% sulfuric acid concentration, 6% solid fraction, and 12.9 min retention time. Under these conditions, the total sugars obtained at 41.8% dry peels and 2.6% of total hexose sugars were further degraded to hydroxymethylfurfural (HMF). No furfural was detected through these experiments from decomposition of pentoses.

Keywords: Orange peel, Dilute-acid hydrolysis, Experimental design, Sugar optimization

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INTRODUCTION

Orange peel, a waste product from citrus processing factories, is partly used for cattle feed. However, the waste has about 66 million tons annual production (Pourbafrani et al. 2007) and a huge amount of it is still discarded to nature, causing several environmental problems (Tripodo et al. 2004). On the other hand, the peel contains various carbohydrate polymers, which make it an interesting choice for production of metabolites such as ethanol by appropriate microorganisms. An individual or combination of mechanical, chemical, and biological pretreatments, however, is required to break down cellulose, hemicellulose, and pectin polymers present in the cell walls of orange peels and convert them to their sugars' monomers (Grohmann et al. 1995; Grohmann and Baldwin 1992; Grohmann et al. 1994).

Enzymatic hydrolysis is an efficient method to release almost all carbohydrates present in the orange peels, but its application is hampered by the high cost of enzymes and the slow rate of the depolymerization reaction (Grohmann et al. 1995). Thus, development of a cost-effective method in which all or a high proportion of carbohydrates could be released will help to commercialize the processes using orange peels as raw materials. The advantages of dilute-acid hydrolysis for peel liquefaction and releasing carbohydrates prior to enzymatic treatment have already been studied (Grohmann et al. 1995; Vaccarino et al. 1989a). There are different variables that might have impacts on the rate and extent of citrus peel hydrolysis by dilute-acid processes. Temperature, acid concentration (or pH), total solid fraction (TS), and time duration of the hydrolysis are the key variables in this process (Grohmann et al. 1995).

Statistical approaches in experimental design provide powerful tools to study and optimize several factors in a process simultaneously. Full factorial, partial factorial, and central composite rotatable designs (CCRD) are the most common techniques used for process analysis and modeling (Montgomery 2001). When the number of factors and responses increases, the last method (CCRD) is preferred, since it needs fewer tests than the other methods and gives almost as much information as other methods (Obeng et al. 2005). This method has already been applied for hydrolysis of a wide variety of materials to find the optimum conditions for corresponding processes (Canettieri et al. 2007; Rahman et al. 2007; Kunamneni and Singh 2005; Rodriguez-Nogales et al. 2007).

Factors that have previously been applied to investigate and optimize the hydrolysis of orange peels were limited to temperature and acid concentration, using one factor at a time rather than varying them simultaneously. This procedure does not allow studying the interaction between variables (if there is any), and thus the best condition that gives the highest yield of monomeric sugars and lowest yield of by-products cannot be achieved. Furthermore, decomposition of pentoses and hexoses produced to hydroxymethylfurfural (HMF) and furfural through the secondary hydrolysis reactions has not been studied.

The scope of the present work was to apply the central composite rotatable design to evaluate the variables that show significant effects in dilute-acid hydrolysis of orange peel. The main and interaction effects of variables on the yields of total liberated sugars and formation of HMF were studied. Based on the experimental design, a model was developed and the optimum conditions to attain the highest yield of carbohydrates and lowest yield of HMF were predicted and validated by an additional experiment. Furthermore, pectin resistance at high temperatures during acid hydrolysis was particularly investigated.

MATERIALS AND METHODS

Substrates and Enzymes

The orange peels used in this work were the residuals of Spanish orange obtained from Brämhults juice AB (Borås, Sweden) and stored frozen at -20 °C until use. The frozen peels were thawed and ground with a food homogenizer (ULTRA-TURAX,TP 18-20, Janke & Kunkel Ika-Labortechnik, Germany) to less than 2 mm in diameter. Total dry content of orange peel was determined by drying at 110 °C for 48 h. Three commercial enzymes, pectinase (Pectinex Ultra SP), cellulase (Celluclast 1.5 L) and β - glucosidase (Novozym 188), were provided by Novozymes A/S (Bagsvaerd, Denmark). Pectinase activity was measured by hydrolyzing 0.02% citrus pectin (P9135-Sigma) solution at 45 °C in 50 mM sodium acetate buffer at pH 4.8 (Wilkins et al. 2007). Cellulase activity was determined by hydrolyzing Whatman#1 filter paper in 50 mM sodium acetate buffer at pH 4.8 and temperature of 45°C (Decker et al. 2003). The activities of pectinase and cellulase were measured as 283 international units (IU)/mg protein, and 0.12 filter paper units (FPU)/mg protein, respectively. The activity of β-glucosidase was reported as 2.6 IU/mg solid by the supplier.

Enzymatic and Acid Hydrolyses

Ground peels were added into 250 ml conical flasks containing 50 mM sodium acetate buffer at pH 4.8 to obtain 100 ml of peel/water slurry with solid fractions of 2, 4, 6, 8, and 10%. The slurries were then hydrolyzed by the enzymes at 45 °C and 140 rpm for 24 h in a shaker bath. For acid hydrolysis, ground peels were diluted with distilled water to obtain 100 ml of peel/water slurry with similar solid fractions as previously mentioned. Sulfuric acid (98%) was added to the slurries to reach final acid concentration of 0, 0.25, 0.5, 0.75, and 1% (v/v). Next, the slurries were heated in an autoclave at various temperatures of 100, 108, 116, 124, and 132 °C with different residence times of 5, 10, 15, 20, and 25 min according to the design of the experiment (Table 1). All samples were then cooled down to 85(±3) °C before removing them from the autoclave.

Acid Hydrolysis at High Temperature

A 10-L high-pressure reactor (Process & Industriteknik AB, Sweden) was used for acid hydrolysis. The reactor heated with direct injection of 60 bar pressure steam, which was provided from a power plant located in Borås, Sweden. The steam hydrolyzed the materials at desired temperature within the designed retention time. The materials were then explosively sent to an expansion tank to cool down for further processing and analyses. In these tests, two kilograms of orange peel/water slurry with solid concentration of 5-18% and acid concentration of 0-0.5% were loaded into the reactor and hydrolyzed for 10-30 min. at 140-210 °C.

Analytical Methods

An ion-exchange Aminex HPX-87P column (Bio-Rad, USA) was used at 85 °C for measuring glucose, galactose, arabinose, and fructose concentrations. Ultra-pure water was used as eluent at a flow rate of 0.6 ml/min. Concentrations of furfural, HMF and galacturonic acid were determined by an Aminex HPX-87H column (Bio-Rad, USA) at 60 °C using 5mM H_2SO_4 at a flow rate of 0.6 ml/min. A refractive index (RI) detector (Waters 2414, Milipore, Milford, USA) and UV absorbance detector at 210 nm (Waters 2487) were used in series. Furfural and HMF concentrations were analyzed from UV chromatograms, whereas the rest of the chemicals were quantified with a refractive index (RI) detector.

Statistical Analysis

The central composite rotatable experimental design method (CCRD) was chosen to determine the effect of four operating variables of the acid hydrolysis, including temperature (T), time, solid (TS), and acid concentration, and two response variables which were (a) yield of sugars defined as the sum of glucose, fructose, arabinose, and galactose produced per total peel dry mass, and (b) conversion of hexoses to HMF, hereafter called HMF yield. Selection of the factors and range of the variables were based on the operating condition, which has a significant influence on the acid hydrolysis process according to previous works (Grohmann et al. 1995; Vaccarino et al. 1989b), as well as 40 preliminary experiments with 110-210 °C, 5-20%(w/w) solid concentration, 0-1%(v/v) acid concentration, and 5-30 min (data not shown).

Table 1: Coded (x_1, x_2, x_3 and x_4) and Respective Actual Levels (T, Solid%, Acid% and Time) in Experimental Design for Dilute-acid Hydrolysis of the Orange Peels by CCRD Method

Test no.	Coded level of variables				Actual level of variables			
	x_1	x_2	x_3	x_4	T(°C)	Solid(%)	Acid(%)	Time(min)
1	-1	-1	-1	-1	108	4	0.25	10
2	+1	-1	-1	-1	124	4	0.25	10
3	-1	+1	-1	-1	108	8	0.25	10
4	-1	-1	+1	-1	108	4	0.75	10
5	-1	-1	-1	+1	108	4	0.25	20
6	+1	+1	-1	-1	124	8	0.25	10
7	+1	-1	+1	-1	124	4	0.75	10
8	+1	-1	-1	+1	124	4	0.25	20
9	-1	+1	+1	-1	108	8	0.75	10
10	-1	+1	-1	+1	108	8	0.25	20
11	-1	-1	+1	+1	108	4	0.75	20
12	+1	+1	+1	-1	124	8	0.75	10
13	+1	+1	-1	+1	124	8	0.25	20
14	+1	-1	+1	+1	124	4	0.75	20
15	-1	+1	+1	+1	108	8	0.75	20
16	+1	+1	+1	+1	124	8	0.75	20
17	-2	0	0	0	100	6	0.5	15
18	+2	0	0	0	132	6	0.5	15
19	0	-2	0	0	116	2	0.5	15
20	0	+2	0	0	116	10	0.5	15
21	0	0	-2	0	116	6	0.0	15
22	0	0	+2	0	116	6	1.0	15
23	0	0	0	-2	116	6	0.5	5
24	0	0	0	+2	116	6	0.5	25
25	0	0	0	0	116	6	0.5	15
26	0	0	0	0	116	6	0.5	15
27	0	0	0	0	116	6	0.5	15
28	0	0	0	0	116	6	0.5	15
29	0	0	0	0	116	6	0.5	15
30	0	0	0	0	116	6	0.5	15

The number of tests required for CCRD is the sum of 2^k factorial runs with its origin at the center, 2k axial runs, and numbers of replicate tests at the center, where k is the number of the variables. In the current work, where the effects of four variables are to be evaluated, the recommended number of tests at the center point is six (Box and Hunter 1957), and therefore the total number of tests would be 30 as presented in Table 1. The values of the variables are coded to lie at ±1 for factorial points, 0 for the center points and ±2 for axial points (Obeng et al. 2005).

A software package called MINITAB® was used to evaluate and to fit the second-order model to these four independent variables according to the following equation:

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^{\ 2} + \sum_{i}^{i \prec j} \sum_{j} b_{ij} x_i x_j + e \qquad (1)$$

where Y is the dependent or response variable(s) to be modeled, x_i and x_j are the independent variables (factors), and b_i, b_{ii} and b_{ij} are the measures of the x_i , x_i^2 and $x_i x_j$ effects, respectively. The variable $x_i x_j$ represents the first-order interactions between x_i and x_j,and e is the error. When the response data are obtained from the test work, a regression analysis using the least-squares method is carried out to determine the coefficients of the response model, their standard errors, and significance. The effects were considered to be not statistically significant when the p-value was higher than 0.05 at the 95% confidence level (Obeng et al. 2005). The optimum values of the selected variables were obtained from the estimated variables in the model and by inspecting the response surface contour plots and MINITAB® optimizer.

RESULTS

Characterization of the Orange Peels

The dry matter content of the orange peels was measured at 20(±1.2)%. In order to find out the carbohydrate content of the peels, they were enzymatically hydrolyzed in shake flasks at 45 ˚C for 24 h. The respective loadings of pectinase, cellulase, and β-glucosidase were 1163 IU/g, 0.24 FPU/g, and 3.9 IU⁄g peel dry matter based on the optimized values previously reported (Wilkins et al. 2007). Yields of various sugars released in the enzymatic hydrolysis were not significantly influenced by increasing the concentration of peel solids. The average values of duplicate experiments are summarized in Table 2. The main carbohydrates after enzymatic hydrolysis were glucose, fructose, galactose, arabinose, and galacturonic acid (GA), accounting for 72% of the total solid content of the peels. These results are in agreement with those obtained from enzymatic hydrolysis of Argentina orange peel (Pourbafrani et al. 2007).

Table 2: Yields of the Sugars Released during Enzymatic Hydrolysis of the Orange Peels

Sugars	(%) of Total Solid
Glucose	27.1 ± 1.3
Fructose	14.1 ± 1.5
Galactose	5.0 ± 0.5
Arabinose	7.1 ± 0.6
Galacturonic acid	18.7 ± 1.5
Total	72

Dilute-acid Hydrolysis

Dilute-acid hydrolyses of the orange peels were performed according to the experimental design presented in Table 1. The results for yields of total sugars (Y_{TS}) and HMF (Y_{HMF}) are displayed in Table 3. The highest yield of sugars, 43.24%, was achieved in the 28th experiment at the center point of the experiments where 116 °C, 0.5% sulfuric acid, 6% solid fraction and 15 min were applied. However, the more reliable value is the average of results of the 25th to 30th experiments, with a Y_{TS} value of 42.40±0.53 %. Furfural, a decomposition product of pentoses, was not detected in any of the experiments, while HMF was the main by-product. The highest yield of HMF was obtained at 9.03% when temperature, time, acid concentration, and solid fraction were 124°C, 20 min, 0.75% and 4%, respectively.

Table 3: Actual Values and Predicted Values for Sugar and Hydroxymethyl Furfural (HMF) Yield

Test no.	Y_{TS}[a] Actual	Y_{TS} Predicted	Y_{HMF}[b] Actual	Y_{HMF} Predicted
1	36.61	33.61	0.75	1.05
2	41.64	40.58	2.35	2.33
3	27.74	27.24	1.17	1.19
4	39.56	40.74	1.74	1.58
5	39.33	36.22	0.95	1.11
6	40.02	38.27	1.54	1.67
7	41.24	39.64	6.67	7.10
8	40.11	39.25	2.70	3.21
9	39.43	38.41	1.42	1.28
10	31.12	30.88	0.77	0.70
11	41.31	41.19	2.48	2.71
12	38.80	41.32	6.12	6.00
13	39.74	37.96	1.80	2.00
14	36.20	36.10	9.03	9.05
15	39.42	39.89	1.80	1.86
16	37.74	38.86	7.33	7.39
17	30.10	32.02	0.81	0.81
18	37.43	37.96	8.06	7.64
19	40.00	43.10	4.07	3.53
20	40.15	39.49	1.90	2.02
21	24.70	29.60	0.00	0.00
22	40.07	37.62	5.50	5.50
23	38.69	40.07	2.32	2.29
24	39.15	40.21	4.14	3.75
25	41.79	42.40	2.65	2.63
26	42.78	42.40	2.61	2.63
27	42.60	42.40	2.64	2.63
28	43.24	42.40	2.67	2.63
29	42.29	42.40	2.63	2.63
30	41.75	42.40	2.60	2.63

[a] Gram of total sugars per 100 gram initial dry matter

[b] As percentage of total hexoses converted to HMF

Dilute-acid Hydrolysis at High Temperature

Pectin was not hydrolyzed under the above hydrolysis conditions and no galacturonic acid was obtained in the hydrolyzates. Therefore, further experiments at higher temperature (140, 180, and 210 °C) for 10 and 30 min. and acid concentrations of zero, 0.05, and 0.5% and solid concentrations of 5, 6, 10, and 18% were carried out. However, no hydrolysis of pectin and no galacturonic acid in the hydrolyzates were obtained. Furthermore, hydrolysis under these conditions did not improve Y_{TS} values than the results reported in Table 3.

Statistical Analysis of the Experimental Results

The results of statistical analysis including the estimated values of factors' coefficients, interactive terms, *t* from Student's *t*-test, and *p*-values are shown in Table 4. The larger magnitude of the *t*-value and the smaller magnitude of the *p*-value indicate more significance of the corresponding coefficient. Thus, temperature and acid concentration in linear and quadratic form were highly significant for the yield of total sugars ($p< 0.05$). Among the interactive terms, only interaction between temperature and acid concentration (X_{13}) was highly significant. For the second response variable (Y_{HMF}), temperature and acid concentration had the greatest effect on the formation of HMF, followed by total solid concentration and time. Temperature was the only significant quadratic term, but it showed interaction with all three other variables. Furthermore, there was an interaction between time and acid concentration regarding the yield of HMF (X_{34} in Table 4).

Table 4: Model Coefficients Estimated by Multiple Linear Regressions for Sugars and Hydroxymethyl Furfural (HMF) Yields

Factor	Yield of sugars			Yield of HMF		
	Coefficient	t-value	p-value	Coefficient	t-value	p-value
Intercept	42.41	42.70	0.000	2.63	19.95	0.000
X_1	1.48	2.99	0.009	1.70	25.86	0.000
X_2	-0.90	-1.82	0.089	-0.37	-5.72	0.000
X_3	2.00	4.04	0.001	1.48	22.45	0.000
X_4	0.03	0.07	0.944	0.36	5.51	0.000
X_1^2	-1.85	-3.99	0.001	0.39	6.45	0.000
X_2^2	-0.28	-0.59	0.560	0.03	0.58	0.569
X_3^2	-2.19	-4.73	0.000	-0.02	-0.37	0.717
X_4^2	-0.56	-1.21	0.242	0.09	1.57	0.136
X_{12}	1.01	1.66	0.116	-0.20	-2.47	0.026
X_{13}	-2.02	-3.33	0.005	1.06	13.11	0.000
X_{14}	-0.98	-1.61	0.126	0.20	2.52	0.024
X_{23}	1.00	1.66	0.118	-0.11	-1.37	0.189
X_{24}	0.26	0.42	0.677	-0.13	-1.70	0.110
X_{34}	-0.54	-0.88	0.388	0.26	3.31	0.005

* X_1, X_2, X_3 and X_4 are temperature, solid fraction, acid concentration and time, respectively. X_{ij} represents the first order interactions between X_i and X_j.

Regression analysis based on the coded variables on the experimental data was performed, and coefficients of the second-order models were calculated. Substitution of coefficients calculated and response variables in Eq. (1) resulted in the following empirical equations for yields of total sugars (Y_{TS}) and HMF (Y_{HMF}):

$$Y_{TS} = 42.41 + 1.48 X_1 - 0.90 X_2 + 2.00 X_3 + 0.03 X_4 - 1.85 X_1^2 - 0.28 X_2^2 - 2.19 X_3^2 - 0.56 X_4^2 + 1.01 X_1 X_2 - 2.02 X_1 X_3 - 0.98 X_1 X_4 + 1.00 X_2 X_3 + 0.26 X_2 X_4 - 0.54 X_3 X_4 \quad (2)$$

$$Y_{HMF} = 2.63 + 1.70\,X_1 - 0.37\,X_2 + 1.48\,X_3 + 0.36\,X_4 + 0.39\,X_1^2 + 0.03\,X_2^{\,2} - 0.02\,X_3^2 + 0.09\,X_4^2 - 0.20\,X_1 X_2 + 1.06\,X_1 X_3 + 0.20\,X_1 X_4 - 0.11\,X_2 X_3 - 0.13\,X_2 X_4 + 0.26\,X_3 X_4 \quad (3)$$

The predicted values obtained from the model equations (Eqs. 2, 3) show good agreement with the actual values of both responses (Table 3). The regression for both responses was statistically significant at the 95% confidence level. The results of the second-order response surface model in the form of analysis of variance (ANOVA) for both responses are presented in Table 5. The suitability of the fitness can be checked by determination coefficients (R^2), which were 0.85 and 0.99 for the yields of total sugars and HMF, respectively. The R^2 statistic indicates the percentage of the variability of the optimization parameter that is explained by the model (Fannin et al. 1981) and, therefore, 15% and 1% of the total variations are not explained by the models developed for the corresponding yields of total sugars and HMF, respectively.

Table 5: Analysis of Variance (ANOVA) for Two Quadratic Models of the Total Sugar (TS) and HMF Yields

Responses	Source of Variation	Sum of squares	Degree of freedom	Mean square	F-value	Probability (p)
Y_{TS}	Regressions	489.67	14	34.97	5.91	0.001
	Residual	88.76	15	5.91		
	Total	578.43	29			
Y_{HMF}	Regressions	154.74	14	11.05	105.74	0.000
	Residual	1.56	15	0.10		
	Total	156.3	29			

Effects of Experimental Variables on Hydrolysis Results

The responses for yield of total sugars were depicted as three-dimensional surface plots of two factors, while the other factors were kept at center levels along with their corresponding contour plots in Figs. 1 and 2. The effects of temperature and acid concentration on the yield of sugars, when solid fraction and time were selected at their center points, are shown in Fig. 1a,b. At the lower levels of temperature, increase in acid concentration resulted in higher yield of sugars. Similarly, at low levels of acid concentration, increase in temperature had a positive effect on the sugar yield. However, at the higher levels of both temperature and acid concentration, the yield of sugars declined, certainly due to presence of the strong interaction between these two variables (Table 4). The maximum yield, 42.6%, was attainable only by conducting hydrolysis experiments in a limited region around 117 °C temperature and 0.6% acid concentration. Under these conditions, HMF yield was 3.4% (Fig. 1c). The effect of acid concentration at the lower levels of temperature on the formation of HMF was negligible, but the rate of sugar decomposition to HMF increased sharply with rising temperature as can be observed from closer counter lines at high temperature and acid concentration in Fig. 1c.

The effects of temperature and time on sugar yield are depicted in Fig. 1d,e. The maximum sugar yield, 42.6%, was obtained at 13 min and 120 °C, while the corresponding HMF yield was 3.4% (Fig. 1f). The minimum sugar yield of 27% was obtained when time and temperature were at the lowest levels, *i.e.* 5 min and 100 °C (Fig.

1e). There was an optimum level for time of hydrolysis where the highest yield of sugars could be obtained (Fig. 1,d,e).

The interdependence of temperature and total solid fraction is presented in Fig. 2a,b. In general, increase in total solid concentration led to a decreased yield of sugars. However, the extent of this reduction was lowered as the temperature was raised (Fig. 2a,b). Analysis of the effect of solid fraction and temperature on the second response variable (Y_{HMF}) showed that at the higher temperature, increase of the solid fraction resulted in a lower yield of HMF (Fig. 2c). The effect of variation of acid concentration and solid fraction is to a great extent similar to the effect of temperature and solid fraction, and therefore the same results can be inferred (data not shown). The highest yield of sugars, 43.1%, was achieved at 116 °C and 0.5% acid concentration with the lowest fraction of solid of 2% (Fig. 2b). The yield of HMF was around 3.5% under these conditions (Fig. 2c). Sugars' yield around 40% is attainable by precise adjustment of both temperature and acid concentration at 10% solid fraction (Fig. 2b).

Analysis of the effect of variation of acid concentration and time on sugar yield is presented in Fig. 2d,e. The surface plot for yield of carbohydrates reached a peak with increase in acid concentration and then declined with further increase in acid concentration. The optimum region that yielded maximum liberation of sugars (42.6%) corresponded to a time period of 15 min and acid concentration of 0.625%. The yield of HMF was around 3% in this region, and the rate of formation increased at higher acid concentration (Fig. 2f).

Based on the second-order models, numerical optimization was carried out to maximize the yield of carbohydrates and minimize yield of HMF, using the response optimizer in MINITAB®. The optimal values of test variables were calculated as 12.9 min, 116 °C, 0.5% v/v acid concentration, and 6% solid fraction, with the corresponding yields of 42.3% for carbohydrates and 2.5% for HMF. The validity of these results was confirmed through performing hydrolysis runs in triplicate under optimized conditions. The analyses show that the average yields of total sugars and HMF were 41.8% and 2.6%, respectively. These experimental findings were in close agreement with the model prediction. Sugars released by acid hydrolysis were equal to 79% of total sugars (excluding galacturonic acid) obtained by enzymatic hydrolysis (Table 2).

Analysis of concentration of various sugars released by carrying out hydrolysis under optimum conditions indicated that yields of glucose, fructose, galactose, and arabinose were 75%, 85%, 80%, and 80% of those from enzymatic hydrolysis, respectively. The major proportion of HMF formation resulted from decomposition of fructose, and there was a direct relationship between loss of fructose and formation of HMF when hydrolysis was performed at test variables higher than the optimum values. Thus, solid residue, which is mostly insoluble and crystalline cellulose, contains about 20% of the remaining, unhydrolyzed glucose and minor amounts of other sugars.

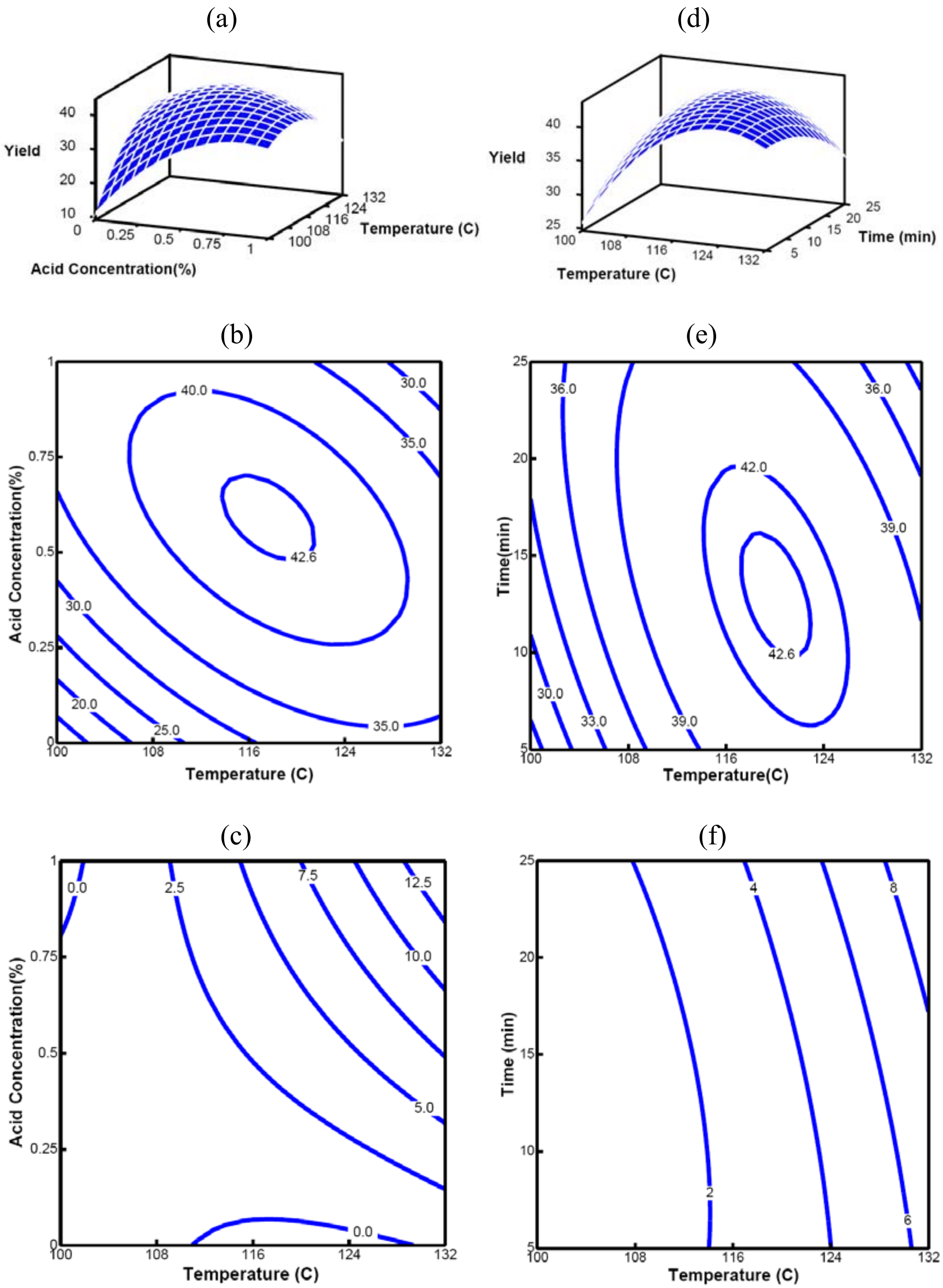

Fig. 1. Effect of temperature and acid concentration, time and temperature on the yield of total sugars (a,b,d,e) and HMF yield (c,f).

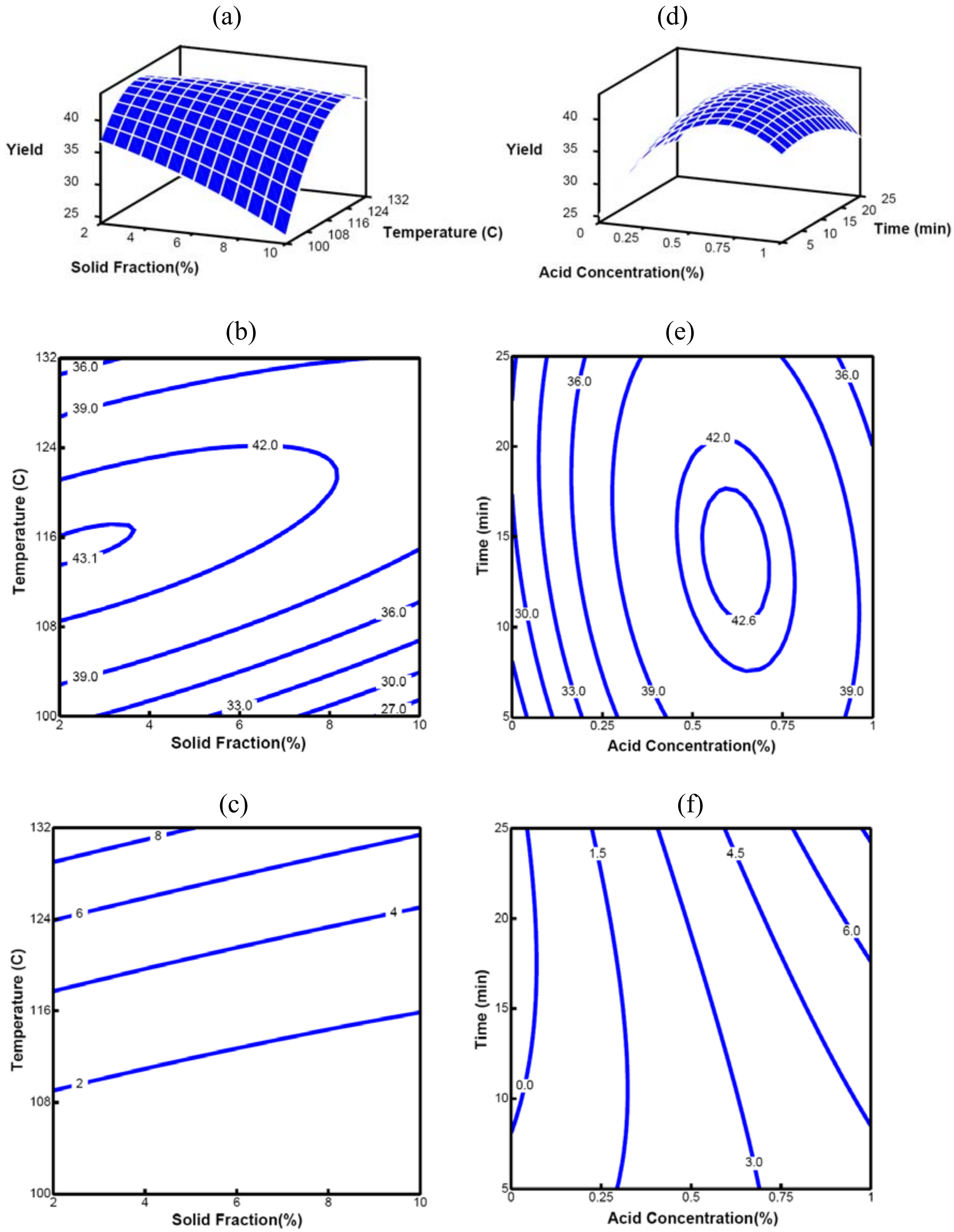

Fig. 2. Effect of temperature and solid faction, time and acid concentration on the sugar yield (a,b,d,e) and HMF yield (c,f).

DISCUSSION

Dilute-acid hydrolysis of citrus peel is an effective treatment prior to enzymatic hydrolysis that allows solubilization of a large fraction of the solid. The remaining residue contains cellulose and solubilized pectin mixed with soluble mono- and oligosaccharides from the peel. The pectin can be either recovered as a product or further hydrolyzed to galacturonic acid and minor amounts of other sugars. A wider range of temperature can be applied in acid than in enzymatic hydrolysis, and the time required for acid hydrolysis is much lower than that for enzymatic reaction (Grohmann et al. 1995). However, acid hydrolysis suffers from the formation of inhibitors such as furfural and HMF due to decomposition of liberated sugars through the secondary reactions (Bienkowski et al. 1987; Taherzadeh et al. 2000; Azhar et al. 1981). Fructose is a soluble sugar in orange peel, which starts to decompose at 120°C, and its rate of decomposition is even faster at higher temperature (Grohmann et al. 1995). These facts suggest that the conditions for dilute-acid hydrolysis and the variables affecting this process should be carefully selected and optimized to yield the highest rate and extent of depolymerization of the carbohydrate polymers and maximum release of sugars, while the formation of inhibitory compounds is minimized.

The results of the current work indicate that furfural was not formed in the range of test variables applied. Furfural is a decomposition product of pentoses, and its formation is a first-order reaction, where the reaction constant is affected by both acid concentration and temperature. Arabinose is the only pentose sugar present in the peel hydrolyzate that is released gradually during hydrolysis. Among the various pentose sugars exposed to the acid for furfural formation, arabinose showed the lowest reactivity, with a small reaction constant (Garrett and Dvorchik 1969). Therefore, lack of furfural formation is most probably due to stability of arabinose and its low concentration in hydrolyzate under the applied conditions.

Formation of HMF during dilute-acid hydrolysis is a sequential reaction where cellulose and hemicellulose are first hydrolyzed to their hexose monomers, followed by decomposition of liberated hexoses to HMF. The kinetics of these two reactions were studied for lignocellulosic materials, and the results indicated that these hydrolysis and decomposition reactions are both first-order reactions and possess rates of similar magnitude (Saeman 1945). These two reaction rates are influenced by temperature and acid concentration. The higher ratio of the first reaction rate constant compared to the second one increases the yield of total liberating sugars. The profile of total liberated carbohydrates vs. time passes through a maximum, and hence there is an optimal time at which sugar production reaches a maximum. This time is a function of constant values of both reactions. Elapsing time of hydrolysis longer than the optimal value enhances the speed of the second reaction, leading to a decrease in net total sugar liberation (Saeman 1945). This fact can be interpreted in another way from results of statistical analysis in Table 4. Time represents no significant effect on the yield of sugars (Y_{TS}), while its effect on the HMF yield is significant and shows interaction with both temperature and acid concentration. Thus, time is an important factor for the overall hydrolysis process to achieve the highest yield of total carbohydrates.

The effect of total solid concentration is significant only for the yield of HMF. The major fraction of HMF is formed from decomposition of the soluble sugars (mainly fructose) that are available in the early period of hydrolysis.Concentration of these soluble sugars is proportional to the solid fraction, and this is a possible reason for the significance of the total solid fraction for the second response variable. The positive impact of total solid fraction on the reduction of HMF yield might be attributed to the presence of temperature and acid concentration gradients at the higher levels of solid fractions, which decreases the decomposition rate of sugars to the HMF in the bulk medium.

Pectin was not hydrolyzed in this work, and therefore no galacturonic acid was detected through the analyses. Despite the soluble nature of released pectin fragments, the glycosidic bonds between galacturonic acid units are probably too resistant to acid hydrolysis due to a combination of inductive and conformational effects. However, because of the low rate of pectin depolymerization, a much longer time of hydrolysis is probably required for partial release of galacturonic acid (Grohmann et al. 1995; Timell et al. 1965).

CONCLUSION

Hydrolysis of citrus processing waste was carried out with dilute acid, and the optimum conditions as well as interaction between influencing factors were investigated by utilizing a central composite rotatable design (CCRD). Among the linear terms, temperature and acid concentration were the most significant variables for both yields of sugars and HMF, and the latter was also influenced by the solid fraction and time. Time of hydrolysis longer than its optimized value proved to have a negative effect on the yield of sugars, mainly due to formation of HMF. At the higher levels of both temperature and acid concentration, total solid fraction had no significant effect on sugar yield. The maximum yield of sugars and minimum yield of HMF can be obtained under the optimum conditions as 41.8% and 2.6%, respectively.

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Paper II

Ethanol Production From Glucose and Dilute-Acid Hydrolyzates by Encapsulated *S. cerevisiae*

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Abstract: The performance of encapsulated *Saccharomyces cerevisiae* CBS 8066 in anaerobic cultivation of glucose, in the presence and absence of furfural as well as in dilute-acid hydrolyzates, was investigated. The cultivation of encapsulated cells in 10 sequential batches in synthetic media resulted in linear increase of biomass up to 106 g/L of capsule volume, while the ethanol productivity remained constant at 5.15 ($\pm$0.17) g/L·h (for batches 6–10). The cells had average ethanol and glycerol yields of 0.464 and 0.056 g/g in these 10 batches. Addition of 5 g/L furfural decreased the ethanol productivity to a value of 1.31 ($\pm$0.10) g/L·h with the encapsulated cells, but it was stable in this range for five consecutive batches. On the other hand, the furfural decreased the ethanol yield to 0.41–0.42 g/g and increased the yield of acetic acid drastically up to 0.068 g/g. No significant lag phase was observed in any of these experiments. The encapsulated cells were also used to cultivate two different types of dilute-acid hydrolyzates. While the free cells were not able to ferment the hydrolyzates within at least 24 h, the encapsulated yeast successfully converted glucose and mannose in both of the hydrolyzates in less than 10 h with no significant lag phase. However, since the hydrolyzates were too toxic, the encapsulated cells lost their activity gradually in sequential batches. © 2005 Wiley Periodicals, Inc.
Keywords: encapsulation; dilute-acid hydrolyzate; furfural; *Saccharomyces cerevisiae*; alginate; ethanol

INTRODUCTION

Fuel ethanol is nowadays a substitute for, as well as additive to, the traditional fossil fuels. Among the different resources used for ethanol production, lignocellulosic materials are the most abundant ones, and they are usually available at low cost. These materials could be processed to the sugar monomers by acid and/or enzymatic hydrolysis followed by fermentation of the sugars to ethanol. Acid hydrolysis is a fast and relatively cheap method for acquiring sugars from lignocelluloses, while enzymatic hydrolysis as well as fermentation usually take long times. On the other hand, acid hydrolysis has the drawback of producing some inhibitors that reduce the fermentability of the resultant hydrolyzates (Luo et al., 2002; Taherzadeh et al., 1997). These inhibitors are furans, phenolic compounds, and carboxylic acids (Clark and Mackie, 1984). Furfural and hydroxymethylfurfural (HMF) are known as two of the strongest inhibitor compounds present in the hydrolyzate. Taherzadeh et al. (1997) found a clear relationship between specific ethanol production rate by *Saccharomyces cerevisiae* in dilute-acid hydrolyzate and the sum of furfural and HMF concentrations. Higher concentrations of furfural and HMF resulted in less fermentability of the hydrolyzates. Banerjee et al. (1981) have shown that furfural at a concentration of 4 g/L inhibited the growth of *S. cerevisiae* and alcohol production by 80% and 97%, respectively.

High cell concentration in the cultivation media can help decreasing the fermentation time as well as increasing the tolerance of the cells against the inhibitors (Chung and Lee, 1985; Galazzo and Bailey, 1990). Immobilization of the cells in alginate has successfully been applied to achieve these goals (Taherzadeh et al., 2001). Among the different methods of immobilization, cell encapsulation is a promising method that probably has advantages over conventional immobilizing methods (Chang et al., 1996; Park and Chang, 2000). In this method, cells are confined in a semipermeable, spherical, and thin membrane. Due to the absence of a solid or gelled core and small wall diameter, mass transfer resistance is less than that of the entrapment methods. The membrane of the capsule should be designed such that the required nutrients and cell products can easily pass through it (Groboillot et al., 1994; Jen et al., 1996). Encapsulation can be carried out by using either natural or synthetic polymers such as calcium alginate (Cheong et al.,

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1993), carrageenan–oligochitosan (Bartkowiak and Hunkeler, 2001), chitosan–CMC (Yoshioka et al., 1990), alginate–poly-L-lysine (Lim and Sun 1980), alginate–oligochitosan (Bartkowiak and Hunkeler, 1999), alginate–aminopropylsilicate (Sakai et al., 2002), agarose (Nigam et al., 1988), and polyamide (Green et al., 1996).

The cell concentration in the capsules can be much higher than in the gel-core beads due to better availability of space. Cheong et al. (1993) reported dry cell concentration of *S. cerevisiae* as high as 309 g/L based on the inside space of the capsule, while the corresponding amount in the calcium alginate beads was less than 50 g/L. A similar phenomenon occurred for *Lactobacillus casei*, where the encapsulated cells in calcium alginate gels yielded higher cell density and productivity than those of gel-core bead (Yoo et al., 1996). Another drawback of the conventional cell entrapment methods, compared to the encapsulated system, is that the cells in large beads grow only in the periphery of the bead because of substrate and eventually oxygen limitation, and therefore can easily leak from the gel matrix and grow in the medium (Park and Chang, 2000; Yamagiwa et al., 1994).

Encapsulation may have other benefits as well. Higher invertase activity of recombinant *S. cerevisiae*, compared to the free cells, was reported in the batch cultivation. Furthermore, invertase in the encapsulated cells showed better thermal stability than in the free cells. At 65°C, the enzyme activity in encapsulated cells lost 35% of its initial activity, whereas the corresponding value for the free cells was 77% (Chang et al., 1996). The long-term operational stability of encapsulated whole-cell enzyme was reported, where the encapsulated whole-cell β-galactosidase using *Escherichia coli* showed gradually increased activity after 30 batches (Oh and Park, 1998). Mei and Yao (2002) reported that the ethanol productivity of encapsulated *S. cerevisiae* during the repeated batch reached a maximum rate of 66.4 g/L·h which was seven times more than that in the free-cell culture.

The main goal of the present work was to investigate ethanol production from dilute-acid hydrolyzates by encapsulated *S. cerevisiae*. Therefore, the experiments were carried out in synthetic medium and glucose as carbon and energy source, followed by investigation of the effect of furfural. Finally, the performance of encapsulated cells was examined in two different types of hydrolyzates. Similar experiments by the free cells were used as references for the effect of encapsulation.

MATERIALS AND METHODS

Dilute-Acid hydrolyzates

The hydrolyzates used in the experiments were produced in a two-stage dilute acid hydrolysis of Swedish forest residuals, originating mainly from spruce trees. The wood chips were loaded into a 350-L rebuilt masonite gun batch reactor located in Rundvik (Sweden), and the first stage of hydrolysis was carried out in 0.5% H_2SO_4 at 12 bar for 7–10 min with 30% (w/w) of solid concentration. The solid residue was then separated from the liquid hydrolyzate by filtration. The second-stage hydrolysis of remaining solid was carried out at a pressure of 21 bar for 7 min. The hydrolyzate liquids were stored at 4°C before use. The compositions of the hydrolyzates are presented in Table I.

Yeast Strain and Medium

The yeast *S. cerevisiae* CBS 8066, obtained from Centraalbureau voor Schimmelcultures (Delft, The Netherlands) was used in all experiments. The strain was maintained on agar plates made from yeast extract 10 g/L, soy peptone 20 g/L, and agar 20 g/L with D-glucose 20 g/L as an additional carbon source. The medium used was defined synthetic medium which has previously been reported (Taherzadeh et al., 1996), with the exception of glucose, which was replaced by hydrolyzates in some of the experiments and furfural was added in some other experiments.

Microencapsulation Method

The cells were prepared in 100-mL volume by growing in cotton-plugged flask in synthetic medium for 24 h. The cells were then separated from the medium by centrifugation at 4,000 rpm for 4 min and resuspended in 100 mL of 1.3% (w/v) sterile $CaCl_2$ solution containing 1.3% (w/v) carboxymethylcellulose (CMC). This solution was added dropwise into the 0.6% (w/v) sterile sodium alginate solution containing 0.1% (v/v) Tween 20 to create the capsules with mean diameter of 3.9–4.2 mm. The alginate solution was stirred at 330 rpm by a magnetic bar. CMC was added to increase the viscosity of the first solution to form spherical capsules, and Tween 20 was used as a surfactant to improve the permeability of the capsule membrane; otherwise the capsules may be ruptured due to formation of CO_2 during the fermentation (Chang et al., 1996). After 10 min of gelation, the capsules were washed with distilled water for 10 min and hardened in 1.3% (w/v) $CaCl_2$ solution for 20 min. The

Table I. Composition of dilute-acid hydrolyzate stages 1 and 2.

	Concentration (g/L)	
Component	Stage 1	Stage 2
Glucose	6.38	19.86
Mannose	14.55	2.80
Xylose	7.68	2.22
Galactose	3.36	0.10
Furfural	0.39	0.31
HMF	0.74	1.58
Acetic acid	4.95	1.35

resultant capsules were then cultured in the medium containing 5 g/L $CaCl_2$ which was added to each flask in order to prevent the capsules from being swelled as previously reported by Chang et al. (1996).

Batch Cultivation Experiments

Prior to the anaerobic cultivations, a total volume of 30 mL of cell-seeded capsules was aerobically cultured in 100 mL of defined growth medium with a glucose concentration of 50 g/L in order to prepare a high biomass concentration inside the capsules. Anaerobic cultivations were then carried out in 300 mL conical flask placed in a shaker bath at 30°C. The liquid volume was 100 mL with 30 mL of cell-seeded capsules, and the shaker speed was 130 rpm. Each flask was equipped with a thick silicone rubber stopper, two stainless steel capillaries, and a glass tube with a loop trap as previously described by Taherzadeh et al. (1997). The capillaries were used for drawing samples with a syringe and injecting nitrogen gas (with less than 5 ppm O_2). The experiments were carried out with four different media: synthetic medium as previously described by Taherzadeh et al. (1996), the synthetic medium containing 5 g/L furfural and the two different hydrolyzates. In order to work with the hydrolyzates, their pH was first adjusted to 5 by addition of 10% $Ca(OH)_2$. Then, the appropriate amounts of all the mineral salts and the trace elements were added to make the same composition as in the synthetic medium, except for the carbon source which was already present in the hydrolyzates.

Analytical Methods

Samples withdrawn from the flasks during the experiments were initially centrifuged and stored at −20°C. Mixture of the sugars including xylose, galactose, and mannose was analyzed by ion-exchange Aminex HPX-87P column (Bio-Rad, Hercules, CA) at 85°C. Ultrapure water was used as eluent at a flow rate of 0.5 mL/min. Glucose, ethanol, glycerol, furfural, HMF, acetic acid, succinic acid, furfuryl alcohol, and furoic acid concentrations were determined by Aminex HPX-87H column (Bio-Rad) at 60°C with 5 mM H_2SO_4 as eluent at flow rate of 0.5 mL/min. A refractive index (RI) detector (Waters 410, Millipore, Milford, MA) and a UV absorbance detector (Waters 486) were used in series. Furfural, HMF, furfuryl alcohol, and furoic acid concentrations were determined from the UV chromatograms at 210 nm, whereas the rest metabolites' concentrations were determined from the RI chromatograms.

Cell-Dry Weight Determination

In order to estimate the cell concentration inside the capsules, the cell-dry weight was measured in different batches. At the end of each batch, 10 capsules were separated and dissolved in 10 mL of 0.1 M sodium citrate solution. The resultant cell-containing solution was centrifuged at 4,000 rpm for 2 min. The samples were then washed by distilled water and dried for 48 h at 110°C. Duplicate measurements were carried out on each sample.

Carbon Balance Calculations

A biomass composition of $CH_{1.76}O_{0.56}N_{0.17}$ was used in the carbon balance calculations (Verduyn et al., 1990). Carbon dioxide produced was considered at the same molar ratio as ethanol. The metabolites and biomass yield were calculated from the determined concentrations at the end of the exponential growth phase. The average carbon recovery determined for 10 different batches was 1.00 with a standard deviation of 3.8%.

RESULTS

Cultivation of Encapsulated Cells in Synthetic Medium

Anaerobic cultivation of encapsulated and free-cell *S. cerevisiae* in synthetic medium was characterized and compared, and the most important results were presented in Figure 1 and Table II. Glucose was used as carbon and energy source throughout all these experiments. The initial cell mass was provided aerobically by cultivation for 24 h in shake flasks. The experiments with the encapsulated cells contained 725 ± 25 capsules in a total volume of ca. 130 mL, and two batches were carried out per day. The capsules were separated at the end of each batch and moved aseptically to a new medium for the next experiment. These experiments were repeated until 10 batches.

The biomass concentration at the beginning of the first batch was 3.14 g/L based on total medium volume, which was equal to 16.22 g/L based on capsule volume. The biomass concentration was increased almost linearly through 10 batches and reached 20.47 g/L of medium or 105.76 g/L capsules (Table II). Therefore, the average calculated yield of biomass per glucose in these 10 batches was 0.043 g/g. This means the biomass inside the capsules was increased by 6.5 times within 10 batches. No cell leakage from capsules to media was observed and the media remained transparent. The CO_2 bubbles formed during the fermentation diffused easily through the capsules' membrane to the medium and left the flasks via loop-trap. Furthermore, the capsules' shapes and diameters remained stable through the 10 batches and were useful for further experiments. At the end of the tenth batch, less than half of the space inside the capsules was filled with yeast cells (Fig. 2). No lag phase in glucose consumption and ethanol production was observed, and fermentation was completed within 5 (±1) h in all 10 batches. These results can be compared with a similar batch experiment with free cells, which was started by similarly prepared cells at

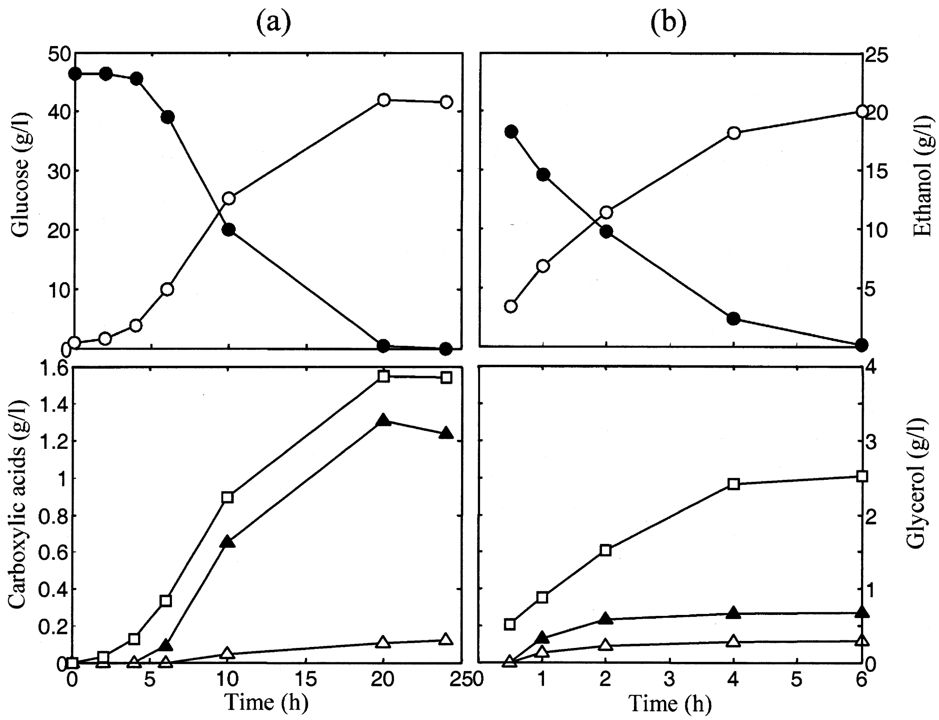

Figure 1. Anaerobic batch cultivation of glucose by (**a**) free cells and (**b**) encapsulated cells in synthetic media: (●) glucose, (○) ethanol, (□) glycerol, (▲) acetic acid, and (△) succinic acid concentrations.

3.14 g/L biomass. The cells in this experiment had about 5 h of lag phase, and 20 g/L sugar was still available in the flask after 10 h (Fig. 1).

The ethanol production rate, glucose consumption rate, and ethanol and glycerol yields in consecutive batches of cultivation of encapsulated cells are presented in Table II. Both glucose consumption and ethanol production rates were several times higher than those of free-cell cultivation. Since the biomass concentration of the first batch of the encapsulated cells was almost equal to the biomass concentration of the experiment with the free cells, the presented data of these two experiments can give a good picture of the advantages of the encapsulation system. The glucose consumption and ethanol production rates were 3.1 and 3.5 times higher with the encapsulated cells, compared to the free cells, respectively. However, increasing of the biomass concentration in the capsules could not increase these rates by more than 60%.

Another advantage of encapsulation is high ethanol and low glycerol yields (Table II). The average yields of ethanol and glycerol of 10 sequential batches with the encapsulated cells are 0.464 and 0.056 g/g. This means the encapsulation has increased the ethanol yield by 9% and decreased the glycerol yield by 30%. A trend similar to that of glycerol was also observed for acetate. The average yield of acetic acid for 10 repeated batches was 0.012 g/g, which

Table II. Key rates and yields in anaerobic batch cultivation of glucose in synthetic media by free cells and 10 sequential batches in encapsulated *S. cerevisiae*.*

Parameter	Free cells	Sequential batch numbers of the cultivations by the encapsulated cells 1	2	3	4	5	6	7	8	9	10
Dry cell[a] (g/L)	3.14	5.43	6.76	8.97	9.99	12.89	14.01	15.94	17.18	19.26	20.48
$-r_S$ (g/L·h)	2.40	7.45	9.37	7.24	8.45	9.02	10.73	11.34	11.82	11.66	10.93
r_E (g/L·h)	0.98	3.45	4.41	3.27	4.20	4.19	4.92	5.27	5.36	5.17	5.07
Y_{SE} (g/g)	0.425	0.465	0.472	0.455	0.499	0.463	0.470	0.465	0.499	0.446	0.463
Y_{SGly} (g/g)	0.079	0.059	0.057	0.054	0.060	0.057	0.060	0.055	0.053	0.052	0.050
Y_{SAce} (g/g)	0.021	0.008	0.006	0.012	0.010	0.010	0.016	0.016	0.016	0.015	0.015
Y_{SSuc} (g/g)	0.003	0.003	0.002	0.003	0.002	0.002	0.003	0.003	0.003	0.003	0.003

*Note: $-r_S$, volumetric glucose uptake rate; r_E, volumetric ethanol production rate; Y_{SE}, ethanol yield; Y_{SGly}, glycerol yield; Y_{SAce}, acetic acid yield; Y_{SSuc}, succinic acid yield.

[a]At the end of exponential growth phase.

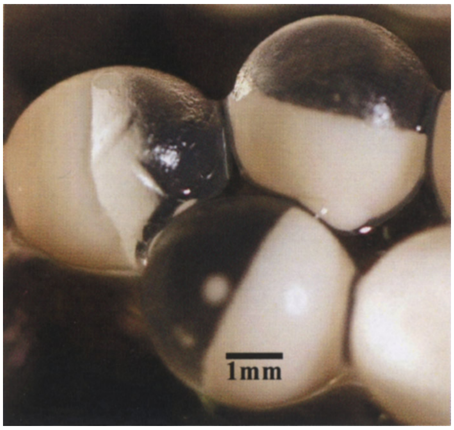

Figure 2. Photograph of capsules containing yeast cells after 10 batches of cultivation.

was much lower than that of free cells, e.g., 0.021 g/g (Table II). The yield of succinic acid by cultivation of the free cells was 0.003 g/g, whereas no significant change in the yield of succinic acid was obtained by encapsulation.

Effect of Furfural on Encapsulated Cells

Furfural is one of the most important inhibitors present in dilute-acid hydrolyzates. The performance and tolerance of encapsulated and free *S. cerevisiae* for this inhibitor were examined. The experiments were carried out anaerobically in shake flasks containing 100 mL of medium including 5 g/L furfural. The experiments with encapsulated cells were carried out in 5 repeated batches in the same manner as the previous set of experiments.

When free cells were exposed to the furfural medium, no growth, sugar consumption, or ethanol production occurred for at least 24 h. However, the encapsulated cells were able to successfully tolerate furfural and take up the sugar with almost no lag phase. The results indicate that the rate of glucose consumption and ethanol production was decreased due to the presence of furfural and the cultivation was completed in longer time (Fig. 3). Although the ethanol productivity was clearly lower than in the corresponding experiments without furfural in all 5 batches, it was still higher than the corresponding value in the free cells without furfural, e.g., 0.98 g/L·h (Fig. 4).

Although the cultivations lasted for more than 20 h, the 5 g/L furfural was converted by the encapsulated yeast in less than 8 h in all the 5 batches (cf. Fig. 3). Furfuryl alcohol was the dominant product of furfural by the encapsulated cells, accounting for 78% of furfural in the first batch (Table III). This value was increased up to 98% of furfural in the fifth batch. Formation of furoic acid was also observed (Table III). However, the concentrations of the acid did not exceed 0.20 g/L in the medium. Formation of 3-(2-furfuryl)-2-hydroxy-2-methyl-3-oxo-propanoic acid (FHMOPA), an acyloin condensation product of furfural and pyruvate, was also detected in small amounts.

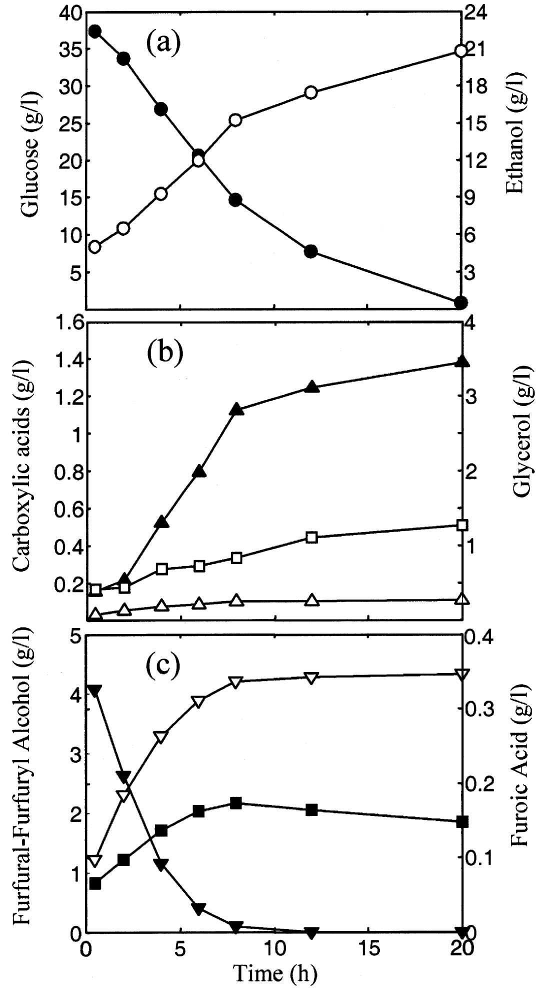

Figure 3. Anaerobic batch cultivation of encapsulated *S. cerevisiae* in synthetic media containing 5 g/L furfural: (**a**) (●) glucose, (○) ethanol; (**b**) (□) glycerol, (▲) acetic acid, (△) succinic acid; (**c**) (▼) furfural, (▽) furfuryl alcohol, and (■) furoic acid concentrations.

The maximum concentration of this compound was higher in the first batch and decreased in the other batches (data not shown).

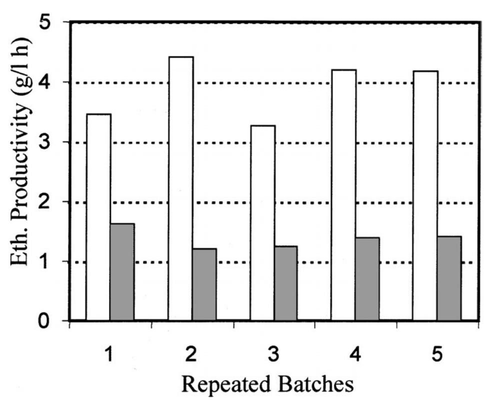

Figure 4. Ethanol production rates in anaerobic batch cultivation of encapsulated *S. cerevisiae* in furfural-free (white bars) and furfural-containing (gray bars) media.

Table III. Key rates and yields in anaerobic cultivation of glucose in synthetic media at the presence of 5 g/L furfural by five sequential batches of encapsulated *S. cerevisiae*.

Batch no.	Cultivation time (h)	Furfuryl alcohol (g/L)	Furoic acid (g/L)	r_E (g/L·h)	Y_{SE} (g/g)	Y_{SGly} (g/g)	Y_{Ace} (g/g)
1	20	3.89	0.20	1.63	0.41	0.030	0.034
2	20	4.81	0.17	1.20	0.41	0.020	0.038
3	21	4.72	0.16	1.24	0.42	0.026	0.045
4	21	4.75	0.15	1.40	0.42	0.020	0.047
5	24	4.91	0.13	1.41	0.41	0.026	0.068

The ethanol yield in furfural-added cultivations was 0.41 ($\pm$0.01) g/g, which is much lower than the corresponding value without furfural added to the media (Table III). The yield of glycerol from the encapsulated cells was highly affected by the addition of furfural to the media. The average glycerol yield of 5 repeated batches was 0.025 ($\pm$0.004) g/g which is less than half of previously reported value for the cultivation in furfural-free media. The acetate yield in the first batch was 0.034 g/g and increased linearly during sequential batches and reached 0.068 g/g in the fifth batch. Hence, the acetate yield obtained in these experiments was significantly higher than the same value in furfural-free cultivation. The presence of furfural had no significant effect on the yield of succinic acid from the encapsulated cells.

Cultivation of Dilute-Acid Hydrolyzates

Cultivation of two different hydrolyzates, stages 1 and 2 (Table I), was investigated in 4 consecutive batch cultivations using encapsulated *S. cerevisiae*, as well as using

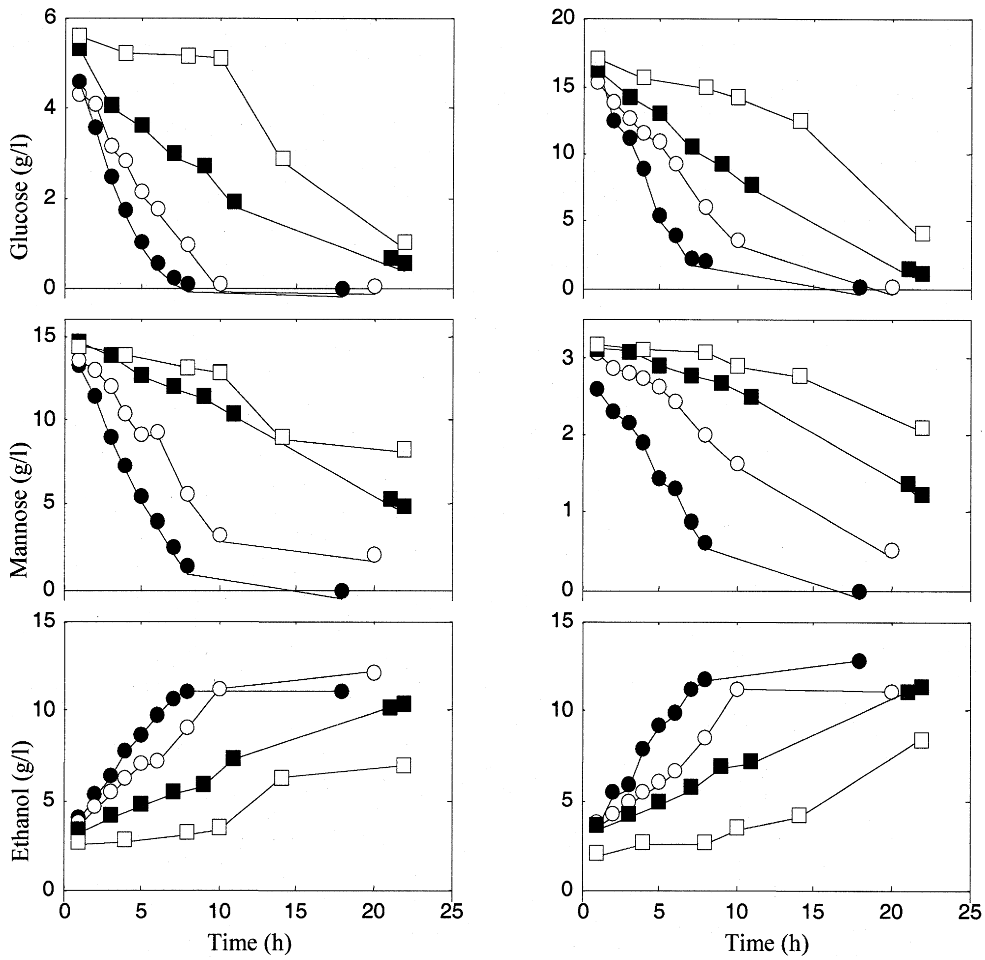

Figure 5. Profiles of glucose, mannose, and ethanol concentrations in consecutive batches of anaerobic cultivation of hydrolyzate stage 1 (left side) and stage 2 (right side) by encapsulated *S. cerevisiae*. Batch numbers: (●) 1, (○) 2, (■) 3, and (□) 4.

Table IV. Anaerobic cultivation of dilute-acid hydrolyzates (stages 1 and 2) by three sequential batches of encapsulated *S. cerevisiae*.

Sequential batch no.	Cultivation time (h)	r_E (g/L·h)		Y_{SE} (g/g)		Y_{SGly} (g/g)	
		Stage 1	Stage 2	Stage 1	Stage 2	Stage 1	Stage 2
1	18	1.108	1.242	0.43	0.45	0.015	0.012
2	20	0.790	0.792	0.42	0.43	0.010	0.016
3	22	0.343	0.375	0.41	0.40	0.006	0.011

free cells. When the 3.14 g/L free cells were exposed to either of the hydrolyzates, no growth, no sugar consumption, and no ethanol production were obtained within 24 h. Thus, the toxicity of the hydrolyzates resulted in either a very long lag phase or a complete failure of the cultivation. However, exposure of the hydrolyzates to the encapsulated yeasts resulted in a quick cultivation with no significant lag phase (Fig. 5).

The profiles of glucose, mannose, and ethanol during cultivation of the two hydrolyzates by encapsulated *S. cerevisiae* are presented in Figure 5. The sugars were consumed at a rate of 2.06 (±0.03) g/L·h in the first batch and ethanol was produced at 1.175 (±0.067) g/L·h (Table IV), which were still higher than the corresponding rates with the free cells in the synthetic medium. These rates were decreased in the second and third batches (Table IV), while a lag phase of a few hours appeared in the fourth batch (Fig. 5). Although the composition of the hydrolyzates was quite different, a similar trend was observed in cultivation of both of the hydrolyzates.

The ethanol yields from the hydrolyzates were less than the corresponding values in the synthetic medium (Table IV). Furthermore, the yields were consecutively reduced in the three batches from 0.43 to 0.41 for the hydrolyzate stage 1 and from 0.45 to 0.40 for the hydrolyzate stage 2 (Table IV). The glycerol yield from cultivation of both of the hydrolyzates was lower than that of synthetic medium containing furfural (Table IV). Furthermore, a stepwise decrease in the yield of glycerol from sequential cultivation of the hydrolyzate stage 1 by the encapsulated yeast was observed (Table IV). Furfural was completely converted in all 4 batches, but the rate of furfural conversion decreased from 0.029 (±0.02) g/L·h in the first batch to 0.016 (±0.01) g/L·h in the last batch. HMF was partially converted in all the batches, e.g., 38% and 27% in the first batches for hydrolyzate stages 1 and 2, respectively. Both the conversion rate and total conversion of HMF were decreased during repeated batches.

DISCUSSION AND CONCLUSIONS

The results of the current study show the advantage of the encapsulated cells in both synthetic media with or without inhibitor and more inhibiting media (hydrolyzate stages 1 and 2). The specific ethanol production rate for the first batches of the encapsulated cells was calculated as 1.10, 0.52, 0.35, and 0.40 g/g·h for the synthetic medium, the synthetic medium with 5 g/L furfural, and the hydrolyzate stages 1 and 2, respectively. However, the corresponding value during the cultivation of the free cells in synthetic medium did not exceed 0.31 g/g·h, when the same concentration of the biomass was used. Furthermore, the presence of the lag phase in the free-cell system contributed to increase the gap between the total fermentation times of the free-cell and encapsulated-cell systems.

The linear increase in biomass concentration during the 10 repeated batch cultivations of the encapsulated cells in synthetic medium to reach the maximum ethanol productivity is a factor that should be considered carefully (Table II). In the series of experiments, the ethanol productivity reached the maximum at about 5 g/L·h after the fifth batch, where the biomass concentration was about 10 g/L based on the total medium volume. However, from batch 6 to 10, the biomass content was doubled, while no increase in ethanol productivity was observed. This fact most likely relates to the mass transfer resistance for the transportation of the sugars to the core of the capsules.

The cells retained in the matrix show some characteristics which are different from the free cells and could be beneficial for the fermentation process. Several previous studies have shown that cell physiology and morphology change upon immobilization. Entrapment of yeast in calcium alginate substantially altered the relative flux control of different steps in the transport and enzymatic pathway in anaerobic fermentation of glucose to ethanol (Galazzo and Bailey, 1990).

The encapsulated cells resulted in higher ethanol yield compared to the corresponding yield from the free cells (Table II). This higher ethanol yield is most likely at the expense of lower concentration of acetic acid and biomass by the encapsulated cells. A comparison between the cultivation by the free cells and the first batch of encapsulated cells may help in understanding this fact (Table II). These two experiments were carried out at identical conditions, with the exception of the encapsulation of the cells. The experiment with the encapsulated cells yielded acetic acid as 0.008 g/g, while it was 0.021 g/g for the experiment with the free cells (Table II). Furthermore, the encapsulated and free cells produced 5.43 and 5.62 g/L biomass in these experiments respectively. On the other hand, the NADH balance for this strain under anaerobic conditions showed a demand of 1.54 and 1.08 g glycerol for each gram of the produced acetic acid and biomass, respectively (Taherzadeh et al., 1996, 2002). Therefore, the experiment with the free cells should have a higher yield by 0.020 g/g glycerol to compensate the extra NADH produced due to the extra yield of 0.013 g/g for the acetic acid compared to the encapsulated cells. A similar calculation for the biomass shows an extra demand of 0.004 g/g glycerol for the difference in biomass production between the two cultivations. This means that if we have a glycerol yield of 0.079 g/g for the free-cell system, we should have a glycerol yield of 0.055 g/g for the encapsulated cells in the first batch. This value is not far from the experimental value of 0.059 g/g (Table II). It can be concluded here that the lower yields of acetic acid, biomass and glycerol from the encapsulated-cell system contribute to increase the yield of ethanol compared to the free-cell system.

The effect of furfural in increasing the lag phase or even failure of the cultivation by *S. cerevisiae* is already known (Banerjee et al., 1981; Chung and Lee, 1985; Sanchez and Bautista, 1988; Taherzadeh et al., 1999). However, the encapsulated cells were able to tolerate furfural and produced more acetate and less ethanol (Table III). A similar phenomenon was reported for free cells when suddenly exposed to furfural, with the exception of releasing acetaldehyde instead of acetic acid (Taherzadeh et al., 1999). The better tolerance against furfural by encapsulated cells compared to the free cells is most likely related to the lower diffusion rate of furfural through the membrane compared to the high capacity of the cells in conversion of furfural.

The balance of NADH can easily describe the high yield of acetate and low yield of ethanol when furfural was present in the media. Furfural was dominantly reduced to furfuryl alcohol. This process needs NADH, and alcohol dehydrogenase (ADH) is one of the enzymes responsible for this conversion (Modig et al., 2002). The demanded NADH was prepared by further production of acetic acid from glucose or possibly even from ethanol.

Production of ethanol from dilute-acid hydrolyzate by the encapsulated cells was quite successful for the first batch. There was no lag phase and the ethanol productivity was even higher than the cultivation of the synthetic medium by the free cells (Table IV). It probably has the same effect on detoxification of the hydrolyzates as previously reported high inoculum level (Boyer et al., 1992). However, the hydrolyzates were too toxic for the cells, and the cells lost their productivity gradually throughout the sequential batches which is probably because of reducing the cells' viability (Nilsson et al., 2002).

It is concluded that encapsulated *S. cerevisiae* can be considered as a good alternative in ethanol production. The encapsulated cells have several advantages, which include higher productivity, higher ethanol yield, lower yields of byproducts, high cell concentration, no lag phase, better tolerance against inhibitors, and no leakage to the media. However, the strain used in the current work may not be suitable for encapsulation and for consecutive fermentation of toxic dilute-acid hydrolyzates and should be replaced with a more tolerant yeast.

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Paper III

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In situ detoxification and continuous cultivation of dilute-acid hydrolyzate to ethanol by encapsulated *S. cerevisiae*

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Abstract

Dilute-acid lignocellulosic hydrolyzate was successfully fermented to ethanol by encapsulated *Saccharomyces cerevisiae* at dilution rates up to 0.5 h^{-1}. The hydrolyzate was so toxic that freely suspended yeast cells could ferment it continuously just up to dilution rate 0.1 h^{-1}, where the cells lost 75% of their viability measured by colony forming unit (CFU). However, encapsulation increased their capacity for in situ detoxification of the hydrolyzate and protected the cells against the inhibitors present in the hydrolyzate. While the cells were encapsulated, they could successfully ferment the hydrolyzate at tested dilution rates 0.1–0.5 h^{-1}, and keep more than 75% cell viability in the worst conditions. They produced ethanol with yield 0.44 ± 0.01 g/g and specific productivity 0.14–0.17 g/(g h) at all dilution rates. Glycerol was the main by-product of the cultivations, which yielded 0.039–0.052 g/g. HMF present in the hydrolyzate was converted 48–71% by the encapsulated yeast, while furfural was totally converted at dilution rates 0.1 and 0.2 h^{-1} and partly at the higher rates. Continuous cultivation of encapsulated yeast was also investigated on glucose in synthetic medium up to dilution rate 1.0 h^{-1}. At this highest rate, ethanol and glycerol were also the major products with yields 0.43 and 0.076 g/g, respectively. The experiments lasted for 18–21 days, and no damage in the capsules was detected.

Keywords: Encapsulation; *S. cerevisiae*; Ethanol; In situ detoxification; Dilute-acid hydrolyzate

1. Introduction

The low price and abundance of lignocellulosic materials are two of the important factors encouraging research and development for converting lignocellulose to fuel ethanol in the last few decades. The pretreatment and acid/enzymatic hydrolysis of these materials result in some inhibitors including furans, carboxylic acids and phenolic compounds, which make the cultivation complicated (Chung and Lee, 1985; Clark and Mackie, 1984; Larsson et al., 1999; Palmqvist and Hahn-Hägerdal, 2000). Dilute-acid hydrolysis is a sim-

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Nomenclature

C_0	initial concentrations of estimated components, i.e. glucose, furfural, HMF and acetic acid in bulk liquid (mM)
C_t	concentrations of estimated solutes in bulk liquid at time t (mM)
C_{inf}	concentrations of estimated solutes in bulk liquid at equilibrium (mM)
CFU	colony forming units
D	dilution rate (h^{-1})
Fur	furfural
HMF	5-hydroxymethyl-furfural
q_E	specific productivity of ethanol (g/(g h))
r_E	volumetric productivity of ethanol (g/l h)
Y_{SE}	ethanol yield (g/g)
Y_{SGly}	glycerol yield (g/g)
Y_{SAce}	acetic acid yield (g/g)
Y_{SSuc}	succinic acid yield (g/g)

ple and fast method of hydrolysis, but this method suffers from formation of relatively high concentration of the inhibitors (Taherzadeh et al., 1997; Galbe and Zacchi, 2002). These toxic compounds result in either poor fermentability or even complete failure of the cultivation of the hydrolyzates. This problem has been a subject of several investigations including physical, chemical and biological detoxification prior to the fermentation, in situ detoxification during the fermentation, and screening/genetic manipulation to find organisms more tolerant to the inhibitors (Brandberg et al., 2004; Sárvári Horváth et al., 2001; Martin and Jonsson, 2003; Taherzadeh et al., 2001, 1999b).

Baker's yeast has the capacity for in situ detoxification, i.e. converting the inhibitors present in the hydrolyzate to less toxic components (Boyer et al., 1992; Chung and Lee, 1985). This capability was applied to develop a fed-batch process for cultivation of severely inhibiting hydrolyzates (Nilsson et al., 2001; Taherzadeh et al., 1999b). Although this method was successful, it showed a drastic decrease in cell viability as soon as the feed rate or toxicity of the hydrolyzate was increased (Nilsson et al., 2001, 2002). A continuous mode of operation has been of interest to cultivate the hydrolyzates (Eldiwany et al., 1992). However, either complete failure of the cultivation or success only at low dilution rates was reported (Eldiwany et al., 1992; Taherzadeh et al., 2001). This means that the cells have very limited capacity for in situ detoxification of the hydrolyzates. Therefore, one should decrease the toxicity of the cells by prior detoxification or increase the cell content in the bioreactor. Brandberg (2005) attempted the latter by cell recycling, filtration and immobilization in alginate beads, but reported no success in fermentation of the hydrolyzate with dilution rate more than $0.2\,h^{-1}$.

Application of encapsulated-cell system in continuous cultivation has several advantages compared to either free-cell or traditionally entrapped-cell system in e.g. alginate matrix. Encapsulation provides higher cell concentrations than free-cell system in the medium, which leads to higher productivity per volume of the bioreactor in continuous cultivation. Furthermore, the biomass can easily be separated from the medium without centrifugation or filtration. The advantages of encapsulation compared to the cell entrapment are having less resistance to the diffusion through the beads/capsules, some degree of freedom in movement of the encapsulated cells, no cell leakage from the capsules, and higher cell concentration (Cheong et al., 1993; Dembczynski and Jankowski, 2000; Nir, 2002; Park and Chang, 2000; Talebnia et al., 2005).

The current work deals with continuous cultivation of a toxic dilute-acid hydrolyzate by encapsulated *Saccharomyces cerevisiae* at different dilution rates. The results were compared with cultivation of free cells in the hydrolyzates and the encapsulated cells in standard media with no inhibitors. The effects of encapsulation on the viability of the cells and in situ detoxification of the hydrolyzates were specifically noticed.

2. Material and methods

2.1. Yeast strain, medium and hydrolyzates

A diploid laboratory strain of the yeast *S. cerevisiae* CBS 8066, obtained from Centraalbureau voor Schimmelcultures (Delft, the Netherlands), was used in all experiments. The strain was maintained on agar plates made from yeast extract 10 g/l, soy peptone 20 g/l and agar 20 g/l with D-glucose 20 g/l as an additional carbon source. The experiments were carried out using a defined synthetic medium according to Taherzadeh et al. (1996), where either 22–24 g/l glucose or a toxic dilute-acid lignocellulosic hydrolyzate was used as carbon and energy source. The hydrolyzate was prepared from spruce forest residuals in a 350-l rebuilt masonite gun batch reactor located in Rundvik (Sweden). The wood chips were pretreated with 0.5% H_2SO_4 at 12 bar for 7–10 min with 30% (w/w) solid concentration (hydrolyzate stage 1). The solid residues were then filtered from the suspension and subjected to the second-stage hydrolysis at 21 bar pressure for 7 min, which resulted in a solution containing: glucose 19.30 ± 0.1 g/l, mannose 6.55 ± 0.5 g/l, galactose 0.62 ± 0.08 g/l, xylose 3.08 ± 0.1 g/l, acetic acid 2.5 ± 0.3 g/l, furfural 0.37 ± 0.01 g/l and hydroxymethyl furfural 1.35 ± 0.07 g/l. The hydrolyzate used throughout these experiments was that from the second stage hydrolysis. It was stored at 4 °C before use, and the pH was adjusted to 5 prior to cultivation.

2.2. Encapsulation procedure and diffusion through the capsules

The capsules were prepared according to Chang et al. (1996) and Talebnia et al. (2005). In this method, the inoculum's cells were centrifuged and re-suspended in 1.3% $CaCl_2$ solution containing 1.3% carboxymethylcellulose (CMC). This solution was added drop-wise into 0.6% sterile sodium alginate solution containing 0.1% Tween 20 to create the capsules with mean diameter of 3.9–4.2 mm and 0.17 (±0.02) mm in membrane thickness. The capsules were then washed with distilled water and hardened in 1.3% $CaCl_2$ solution for 30 min. Some aseptic capsules were treated in order to measure and compare the diffusion rates of different components through the capsules' membranes. In this test, 200 ml solution containing 50 mM of each of glucose, acetic acid, furfural and HMF at pH 5 was added into a 500 ml conical flask and placed in a shaker bath at 30 °C and 100 rpm. Then, 100 ml of cell-free capsules were added to the flask and the profiles of the concentration of the mentioned chemicals were followed for 120 min.

2.3. Continuous cultivation experiments

The cultivation experiments were initiated by a 20-h aerobic batch cultivation of 250 ml cell-seeded capsules on 50 g/l glucose, in order to prepare biomass inside the capsules. It was then followed by anaerobic continuous cultivations. A bioreactor (Biostat A., B.Braun Biotech, Germany) with 1 l working volume was used in all the experiments and the temperature and pH were controlled at 30 °C and 5, respectively. Nitrogen gas was continuously sparged to the bioreactor at flow rate 0.4 l/min, which was controlled by a mass flow controller (Hi-Tech, Ruurlu, Netherlands). A peristaltic pump (Watson-Marlow Alitea AB, Sweden) was used to feed the bioreactor. Samples withdrawn from the bioreactor were centrifuged and stored at −20 °C.

2.4. Analytical methods

The metabolites were quantified by HPLC. Glucose, xylose, galactose and mannose were analyzed by ion-exchange Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) at 85 °C. The eluent was ultrapure water at flow rate 0.6 ml/min. An evaporative light scattering detector (Waters 2465, MA, USA) was used to quantify the sugars. Ethanol, glycerol, furfural, HMF, acetic acid and succinic acid concentrations were determined by Aminex HPX-87H column (Bio-Rad) at 60 °C with 5 mM H_2SO_4 as eluent at flow rate 0.6 ml/min. A refractive index (RI) detector (Waters 2410, Millipore, Milford, USA) and a UV absorbance detector (Waters 2486) were used in series. Furfural and HMF concentrations were analyzed from the UV chromatograms at 210 nm, whereas the rest of metabolites were analyzed from the RI chromatograms. The standard deviation was less than 2% for all samples analyzed.

The cell viability and vitality were determined using colony forming unit (CFU) and trypan blue staining methods, respectively. The cells were provided from dissolution of 10 capsules in sodium citrate solution at the end of each experiment. For CFU measurements, triplicate samples of 0.1 ml of cell suspension were diluted 10^4–10^6 times and spread on agar plates, which were then placed in the incubator at 30 °C for 48 h. The plates with 30–300 colonies were used for viability estimation. Total cell numbers and vitality were estimated by light microscopy and Bürker counting chamber according to Nilsson et al. (2001) with the exception of using trypan blue instead of methylene blue. The cell dry weight was also measured at different dilution rates, where duplicate samples of 10 capsules were separated, dissolved in 10 ml Na–citrate solution, centrifuged, washed and dried for 48 h at 110 °C. The standard deviation of all duplicated samples had a value of less than 7%.

3. Results

3.1. Continuous cultivation of encapsulated cells in synthetic medium

Continuous cultivation of glucose in synthetic medium by encapsulated *S. cerevisiae* was carried out by 250 ml capsules containing 4.04 g biomass in a total volume of 1 l medium. The feed was a solution of 22–24 g/l glucose supplemented with the other minerals and fed at dilution rates 0.2, 0.4, 0.5, 0.8 and 1.0 h^{-1} into the bioreactor. The feed rates were kept constant for at least five retention times in each dilution rate to be certain of reaching steady-state conditions. The most important results are summarized in Table 1.

The encapsulated yeast cells were able to consume glucose at the tested dilution rates, but increasing the dilution rates resulted in higher residual glucose in the outflow of the bioreactor. While more than 99% of glucose was consumed up to dilution rate 0.4 h^{-1}, the cells left 37% of the glucose at dilution rate 1.0 h^{-1} (Table 1). The ethanol yield from glucose at dilution rate 0.2 h^{-1} was 0.40 g/g, whereas it was 8% higher at 0.435 ± 0.005 g/g for the other rates. The volumetric ethanol productivity was increased from 1.64 to 6.50 g/l h from the lowest to highest dilution rates, while the specific ethanol productivity decreased from a maximum of 0.29 g/(g h) at 0.4 h^{-1} to 0.19 g/(g h) at the highest rate.

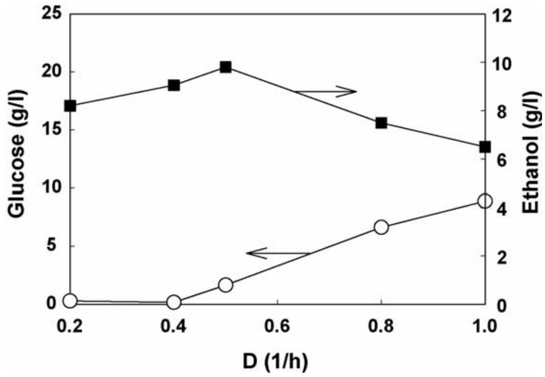

Fig. 1. The steady-state concentration of residual glucose and produced ethanol at different D (h^{-1}) during anaerobic continuous cultivation on glucose using encapsulated yeast cells: (○) glucose and (■) ethanol. The arrows show the corresponding axes for glucose and ethanol concentration.

The steady-state concentrations of glucose and ethanol as a function of dilution rates are presented in Fig. 1. Ethanol concentration increased slightly at lower dilution rates (from 0.2 to 0.5 h^{-1}) and reached 9.8 g/l, while it declined at higher rates. The only important by-product of the encapsulated cells in terms of concentration was glycerol. At dilution rate 0.2 h^{-1}, the glycerol yield showed the highest value of 0.097 g/g, while it decreased slowly to 0.076 g/g at the highest rate 1.0 h^{-1}. Acetic and succinic acids were also detected in trace amounts (Table 1).

3.2. Free cell cultivation in hydrolyzate

Fermentability of the hydrolyzate was investigated using freely suspended *S. cerevisiae* in continuous cultivation. The yeast was first cultivated in defined

Table 1
Summary of the most important results of anaerobic continuous cultivation of encapsulated *S. cerevisiae* on glucose at steady-state conditions

D (h^{-1})	Glucose conversion (%)	Ethanol			Other metabolites		
		Y_{SE} (g/g)	r_E (g/l h)	q_E (g/(g h))	Y_{SGly} (g/g)	Y_{SAce} (g/g)	Y_{SSuc} (g/g)
0.2	100	0.40 ± 0.03	1.64	0.26	0.097	0.005	0.003
0.4	99.2 ± 0.5	0.44 ± 0.01	3.62	0.29	0.080	0.007	0.002
0.5	93.1 ± 0.4	0.44 ± 0.01	4.89	0.22	0.078	0.009	0.003
0.8	72.3 ± 1.5	0.43 ± 0.01	5.99	0.20	0.077	0.007	0.003
1.0	63.0 ± 2.0	0.43 ± 0.02	6.50	0.19	0.076	0.007	0.003

Note: All calculations of yields were based on the consumed glucose.

Table 2
The most important results of anaerobic continuous cultivation of dilute-acid hydrolyzate at steady-state conditions by "freely suspended" *S. cerevisiae*

Parameters	D (h^{-1})	
	0.1	0.2
Hexose conversion (%)	90 ± 2	Wash out
Y_{SE} (g/g)	0.34 ± 0.03	
q_E (g/(g h))	0.53	
r_E (g/(l h))	0.857	
Y_{SGly} (g/g)	0.036	
Furfural conversion (%)	100	
HMF conversion (%)	48 ± 3	
Viability (%)	25 ± 2	

Note: All calculations of yields and hexose conversion were based on the sum of the consumed glucose, mannose and galactose.

medium containing 50 g/l glucose in batch mode of operation, to reach 4.81 g/l dry weight biomass. The hydrolyzate was then fed to the bioreactor at dilution rate 0.1 h^{-1}. The steady-state results after five retention times (50 h cultivation) are presented in Table 2. In this condition, the cell dry weight was measured as 1.61 g/l, which is much less than the initial biomass. Furthermore, the cell viability measured by CFU decreased to 40% and 25% after 2.5 and 5 retention times, respectively. The ethanol yield was 0.34 g/g.

The experiment was then continued by increasing the dilution to 0.2 h^{-1}. However, just after increasing the dilution rate, the carbon dioxide evolution rate decreased drastically and approached zero, fermentation ceased and all cells were washed out from the bioreactor. This is considered to be evidence of the toxicity of the hydrolyzates.

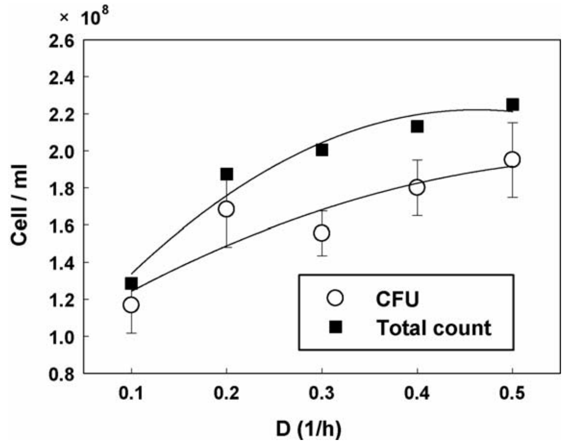

Fig. 2. Total cell count (■) and CFU (◯) results of the increased D (h^{-1}) in anaerobic continuous cultivation of undetoxified dilute-acid hydrolyzate by encapsulated *S. cerevisiae*. (The values of CFU are an average of triplicate measurements with maximum standard deviation of 8%.)

3.3. Continuous cultivation of toxic hydrolyzate by encapsulated cells

Continuous cultivation of the hydrolyzate by encapsulated *S. cerevisiae* was carried out by 250 ml capsules containing 4.02 g biomass in a total volume of 1 l medium. Dilution rates 0.1, 0.2, 0.3, 0.4 and 0.5 h^{-1} were investigated, where the feed rates were kept constant for at least five retention times to reach steady-state conditions. The most important results are summarized in Table 3 and Fig. 2.

The cells assimilated glucose, mannose and galactose, while xylose could not be taken up by the yeast.

Table 3
Summary of the key results of anaerobic continuous cultivation of dilute-acid hydrolyzate by "encapsulated" *S. cerevisiae* at steady-state conditions

D (h^{-1})	Conversion (%)				Y_{SE} (g/g)	Y_{SGly} (g/g)	r_E (g/(l h))	q_E (g/(g h))	Viability (%)
	Glucose	Mannose	Furfural	HMF					
0.1	95 ± 1	98 ± 1	100	71 ± 3	0.44 ± 0.01	0.052	1.146	0.17	90 ± 9
0.2	84 ± 2	87 ± 3	100	57 ± 8	0.45 ± 0.01	0.044	2.053	0.16	90 ± 9
0.3	79 ± 5	79 ± 7	92 ± 5	71 ± 9	0.43 ± 0.02	0.041	2.717	0.14	77 ± 6
0.4	72 ± 6	90 ± 5	75 ± 4	52 ± 7	0.45 ± 0.02	0.041	3.545	0.15	84 ± 7
0.5	71 ± 5	79 ± 4	69 ± 6	48 ± 5	0.45 ± 0.03	0.039	4.206	0.16	85 ± 8

Note 1: Calculations of ethanol and glycerol yields were based on the sum of the consumed glucose, mannose and galactose.
Note 2: Viability was determined as a ratio of CFU and total cell count using light microscopy and Bürker chamber.
Note 3: Vitality estimated by trypan blue staining and light microscopy was more than 90% at all dilution rates.

The yeast was able to ferment hexoses to ethanol at all dilution rates, which is considered a success in cultivation without any prior detoxification (Table 3). While more than 95% of fermentable sugars were assimilated to ethanol at dilution rate $0.1\ h^{-1}$, the cells left 29% of initial glucose at rate $0.5\ h^{-1}$. The average ethanol yield was 0.44 ± 0.01 g/g, which is slightly higher than the corresponding number for fermentation of glucose by encapsulated cells (cf. Tables 1 and 3). The volumetric ethanol productivity (r_E) proportionally increased with dilution rate, while the specific productivity (q_E) was almost constant at 0.16 g/(g h) regardless of the dilution rate (Table 3).

The glycerol produced had the same trend as the cultivation in synthetic medium, i.e. a gradual decrease with increasing dilution rate (Table 3). However, the absolute values of the yields were lower than in the synthetic medium, and on the same order of the cultivation of the free cells in the hydrolyzate (cf. Tables 1–3). The conversions of furfural and HMF were also monitored. Furfural was totally converted and not detected at low dilution rates 0.1 and $0.2\ h^{-1}$, while the cells could not convert it totally at higher rates. On the other hand, the yeast cells were not able to convert HMF by more than 71%, which occurred at low dilution rates (Table 3).

The cell vitality defined by "Trypan blue staining method" at all dilution rates was more than 90% and was not affected or changed by dilution rates (data not shown). The fraction of viable cells estimated by colony forming unit showed a great difference in viability of encapsulated and free cells in cultivation in the hydrolyzate. At the lower dilution rates (0.1 and $0.2\ h^{-1}$) approximately 90% of yeast cells were viable, while at higher dilution rates this value was slightly decreased to 77–85% (Table 3). The viable cells and total cells showed almost the same trend and increased with time and dilution rates. The viable and total cells concentration at dilution rate $0.1\ h^{-1}$ were 1.168×10^8 and 1.287×10^8 cell/ml of medium, which increased at dilution rate $0.5\ h^{-1}$ by 67% and 75%, respectively (Fig. 2).

3.4. Penetration through capsule membrane

The diffusion of glucose, furfural, HMF and acetic acid through the membrane into the interior space of capsules was examined. In this investigation, the capsules contained no cells and the diffusion of these components was followed for 120 min, and the results are presented in Fig. 3. The results show that the membrane had no selectivity in permeating the sugars or the inhibitors during the cultivation. All the components needed 20 and 40 min to reach $90 \pm 3\%$ and $99 \pm 0.5\%$, respectively, of final concentration in equilibrium through the capsules' membranes.

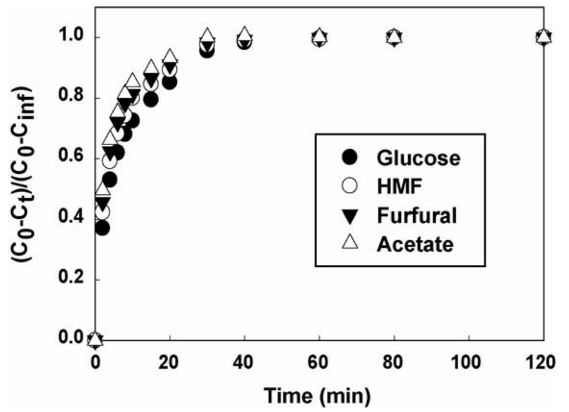

Fig. 3. Profile of the solute diffusion (HMF) through the capsules' membrane from bulk liquid.

4. Discussion

Encapsulation seems to be a promising method to keep the cells viable in toxic environments such as dilute-acid hydrolyzates and help the process to run continuously at high dilution rates and high productivities. Cultivation with encapsulated cells has clear advantages over cultivation with free cells in which the dilution rates higher than $0.1\ h^{-1}$ could not be achieved (Brandberg, 2005), and over cultivation with immobilized cells in traditionally entrapped system in which the toxic hydrolyzates should be first detoxified (Taherzadeh et al., 2001).

Immobilization has advantages compared to free cells in continuous cultivation of lignocellulosic hydrolyzates in providing higher biomass in the bioreactor. Furthermore, it results in a better cultivation even compared to cultivation with cell-recycling system (Brandberg, 2005). While the optimum dilution rate in free-cell cultivation has a high limit of e.g.

$0.15\,h^{-1}$, immobilization removes this limit to achieve a dilution rate of e.g. $1.5\,h^{-1}$ (Nigam, 1999, 2000). However, toxicity of the hydrolyzates is a problem that stops the cultivations with either free or immobilized cells, by drastically deceasing the viability of the cells (Nilsson et al., 2001, 2002). Therefore, detoxification is usually considered in connection with the cultivation of lignocellulosic hydrolyzates (Taherzadeh et al., 2001).

In situ detoxification of the hydrolyzates by *S. cerevisiae* is another alternative to prior detoxification. The yeast was previously shown to be able to convert or assimilate some of the inhibitors present in the hydrolyzates such as furfural, HMF, acetic acid and some phenolic compounds, although the conversion rates of various inhibitors, *e.g.* furfural and HMF are different (Delgenes et al., 1996; Modig et al., 2002; Taherzadeh et al., 1999a, 2000). This capability is a function of cell concentration, growth rate and viability, and the concentration of the inhibitors as well (Chung and Lee, 1985; Delgenes et al., 1996). These factors are valid for in situ detoxification by encapsulated cells. However, there should be another factor that makes the encapsulated cells more tolerant than free and entrapped cells. Using a more tolerant strain of *S. cerevisiae* (ATCC 96581), Brandberg (2005) could not achieve higher dilution rates than $0.14\,h^{-1}$ for the free cells and $0.2\,h^{-1}$ for the immobilized cells in alginate matrix, in cultivation of hydrolyzate identical to this work and lignocellulose hydrolyzate supplemented with wheat hydrolyzate, ammonium sulphate and biotin, respectively. If we compare these results with the current work, where the cells fermented the undetoxified hydrolyzate at dilution rate $0.5\,h^{-1}$ while still more than 75% of the cells were viable, it reveals that the encapsulated cells behave differently than entrapped or free cells. This phenomenon is yet to be more investigated. It may have something to do with growth of the cells in the form of a packed community inside the capsules, while the entrapped cells live almost alone within the matrix. Carboxymethylcellulose (CMC) present in capsules membrane could play a role in giving a better tolerance to the encapsulated cells against the inhibitors. It should also be noticed that permeation of the inhibitors and sugars through the membrane is hardly connected with this phenomenon, since the membrane showed no selectivity in permeation of the compounds.

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Physiological and morphological study of encapsulated *Saccharomyces cerevisiae*

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Abstract

The impact of encapsulation on the anaerobic growth pattern of *S. cerevisiae* CBS 8066 in a defined synthetic medium over 20 consecutive batch cultivations was investigated. In this period, the ethanol yield increased from 0.43 to 0.46 g/g, while the biomass and glycerol yields decreased by 58 and 23%, respectively. The growth rate of the encapsulated cells in the first batch was 0.13 h^{-1}, but decreased gradually to 0.01 h^{-1} within the 20 sequential batch cultivations. Total RNA content of these yeast cells decreased by 39% from 90.3 to 55 mg/g, while the total protein content decreased by 24% from 460 to 350 mg/g. On the other hand, the stored carbohydrates, that is, glycogen and trehalose content, increased by factors of 4.5 and 4 within 20 batch cultivations, respectively. Higher biomass concentrations inside capsules led to a lower glucose diffusion rate through the membrane, and volumetric mass transfer coefficient for glucose was drastically decreased from 6.28 to 1.24 (cm^3/min) by continuing the experiments. Most of the encapsulated yeast existed in the form of single and non-budding cells after long-term application.

Keywords: Encapsulation; Yeast physiology; Ethanol; Glycogen; Proteins; Mass transfer coefficient

1. Introduction

Alternative fuels are nowadays in focus due to global warming caused by excessive emissions of CO_2, to shortage of oil reservoirs and to unstable prices of fossil fuels. Ethanol as a suitable substitute for fossil fuel can be produced from lignocellulosic materials such as wood residuals. However, despite availability and low price, lignocelluloses need hydrolysis and pre-treatment prior to fermentation for ethanol production [1–3].

Despite great progress in more than two decades of research and development, fermentation of lignocellulosic hydrolyzates to ethanol is still a challenge due to formation of many toxic components during the pre-treatment and hydrolysis [4–6]. Yeast cells have a capacity for *in situ* detoxification [4,7] and utilizing this capacity was the basic principle in development of fermentation processes by, for example, fed-batch or continuous cultivations [8,9]. Although much success in fermentation of toxic hydrolyzates was achieved, prior detoxification was still necessary for very toxic hydrolyzates [10]. However, an encapsulated-cell system has demonstrated high capacity for fermenting toxic hydrolyzates with no prior detoxification [9,11]. The yeast cells, after being enclosed inside a membrane, tend to attach to the interior surface of capsules and then start to grow and colonize. It seems that this kind of direct physical contact of cells with each other and with the solid surface, and growing in a limited space, affects the cells' metabolism and modifies their growth pattern [9,12]. There are a few studies on the metabolic parameters of immobilized cells (entrapped/adsorbed in gel matrix/carrier) compared to free cells, where growth rate, yeast energetic metabolism, resistance to inhibitors and carbohydrate content were investigated [12–15]. However, no publications on the metabolic behaviour of encapsulated cells have been noted.

The focus of the current work was to study the morphology of *S. cerevisiae* inside the capsules and to investigate the physiological responses of the encapsulated cell to the altered environmental conditions, with respect to their internal composition changes over long-term application. To get a better understanding of cells' behaviour under encapsulation, the total carbohydrates, RNA, protein, and two important carbohydrate reserves – that is, glycogen and trehalose content of yeast cells – were analysed. The fermentative capacity of encapsulated cells,

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Nomenclature

Y_{SX}	Biomass yield (mg/g)
Y_{SE}	Ethanol yield (mg/g)
Y_{SGly}	Glycerol yield (mg/g)
Y_{SAce}	Acetic acid yield (mg/g)
Y_{SSuc}	Succinic acid yield (mg/g)
Y_{SC}	Carbon dioxide yield (mg/g)
μ	Specific growth rate (h^{-1})
k	Mass transfer coefficient (cm/min)
a	Total external surface area of capsules (cm^2)
K	Volumetric mass transfer coefficient (cm^3/min)
V_0	Volume of solution outside capsules (cm^3)
V_C	Volume of solution inside capsules (cm^3)
C	Solute concentration in solution outside capsules (g/l)
C_C	Solute concentration in solution inside capsules (g/l)
C_{eq}	Solute concentration in the equilibrium state (g/l)

as well as diffusion characteristics of membrane for glucose, was also investigated.

2. Material and methods

2.1. Yeast strain, medium, encapsulation and diffusion test

The yeast *S. cerevisiae* CBS 8066, obtained from Centraalbureau voor Schimmelcultures (Delft, the Netherlands), was used in all experiments. The strain was maintained on agar plates made from yeast extract 10 g/l, soy peptone 20 g/l, and agar 20 g/l with D-glucose 20 g/l as an additional carbon source. The experiments were carried out using a defined synthetic medium according to Taherzadeh et al. [16] where 50 g/l glucose was used as carbon and energy source.

The capsules with mean diameter of 3.2–3.5 mm and thickness of 0.17 mm were prepared based on a method previously reported [11]. Diffusion rate of glucose through the capsules membrane was examined through the experiments. Cell-free capsules, and the capsules after 5, 10 and 20 batch cultivations, were collected and added to a solution containing 50 g/l glucose, where the total volume of the capsules was 25% of the total volume of the solution. The profile of the glucose concentration was followed within 240 min in order to calculate the mass transfer coefficient of glucose.

2.2. Batch cultivation and carbon recovery

A total volume of 50 ml cell-seeded capsules was aerobically cultured in 200 ml of defined growth medium, with 50 g/l glucose as a carbon source in order to provide a high biomass concentration inside the capsules. Sequential anaerobic cultivations were then carried out by separating the capsules and resuspending in 200 ml new culture medium, which was repeated 20 times. The total number of the capsules was about 2300 with a total volume of 50 ml. The cultivations were carried out in a shaker bath at 30 °C and with speed of 130 rpm according to Talebnia et al. [11]. At the end of each cultivation 20–70 capsules were collected, and their membranes were mechanically ruptured; their yeast content was separated, washed twice with NaCl 0.9% and stored in a freezer at −20 °C for further analyses. The supernatants of the cultivations were analysed by HPLC. The balance in carbon recoveries was calculated from the production of the most important metabolites, biomass and CO_2, where carbon dioxide production was assumed to be at the same molar ratio as ethanol.

2.3. Determination of mass transfer coefficient

Encapsulated cells in a medium containing nutrients are assumed to constitute a two-phase system separated by the capsules' membrane. Mass transfer between two phases can be described as [17]:

$$V_0 \frac{dC}{dt} = -ka(C - C_C) \tag{1}$$

where C and C_C are the solute (glucose) concentrations in solution outside and inside the capsules, respectively; k and a are the mass transfer coefficient and total external surface area of capsules; V_0 is the volume of solution outside capsules, and t is time. Mass balance for solute in both phases gives:

$$\frac{d(V_0 C + V_C C_C)}{dt} = 0 \tag{2}$$

Assuming a constant solute concentration in the equilibrium state (C_{eq}) in both phases produces a direct relationship between C and C_C, which can be incorporated in Eq. (1). This equation can be solved with the initial condition of $C_{t=0} = C_0$ to obtain:

$$C = C_{eq} + (C_0 - C_{eq}) \exp\left(-\left[\frac{1}{V_0} + \frac{1}{V_C}\right] kat\right) \tag{3}$$

In this model, it is assumed that there is no concentration gradient in the capsule interior. With the ratio of V_C and V_0 applied in the experiments, which was 1:3, Eq. (3) is expressed as:

$$\frac{C}{C_0} = \frac{C_{eq}}{C_0} + \left(1 - \frac{C_{eq}}{C_0}\right) \exp\left(-4\frac{Kt}{V_0}\right) \tag{4}$$

where $K = ka$ is the volumetric mass transfer coefficient. The values of K in different batch cultivations were determined by fitting a theoretical curve from Eq. (4) to experimental data with a non-linear regression program using MATLAB® in which the root-mean-square error between the experimental data and the model prediction was minimized.

2.4. Analytical methods

Sample solutions withdrawn from the flasks during the experiments were initially centrifuged and stored at −20 °C until use. Glucose, ethanol, glycerol and acetic acid concentrations were quantified by Aminex HPX-87H column (Bio-Rad) at 60 °C with 5 mM H_2SO_4 as eluent at flow rate of 0.6 ml/min. A refractive index (RI) detector (Waters 2414, Millipore, Milford, USA) was used for all mentioned chemicals. The cell-dry weight was measured over sequential batch cultivations as previously reported [11]. The standard deviation of all duplicated samples was less than 8%. The numbers of budding and total cells were estimated by light microscopy and Bürker counting chamber with appropriate diluted cell concentrations.

Total protein content of the cells was determined by addition of 3 ml 1 M NaOH to samples previously washed and stored in the freezer. The pellet was suspended and boiled for 10 min. After cooling on ice, the protein content was measured by the Biuret method [18]. Bovine serum albumin (Sigma, A-7908) was used as a standard. RNA content of the cells was estimated based on a method proposed by Benthin et al. [19], where RNA was extracted by $HClO_4$, degraded by KOH and concentration of the resulted nucleotides were measured by spectrophotometer at 260 nm using the average nucleotide data.

Trehalose and glycogen content of yeast cells were determined according to Parrou and Francois [20]. In this method, the yeast cells are suspended in 0.25 M Na_2CO_3 solution and incubated at 95 °C for 4 h. The pH is then adjusted to 5.2 by a buffer solution and the polysaccharides are hydrolysed overnight with *Aspergillus niger* amyloglucosidase at 57 °C and trehalase at 37 °C. The glucose liberated was determined by enzymatic kit (Boehringer, Cat. No. 10716 251 035). The results were expressed as the percentage of glucose equivalent to glycogen or trehalose per cell dry weight (%w/w).

Total carbohydrate was determined by colorimetric method according to Herbert et al. [14], where phenol-sulfuric acid reagent was applied. The absorbance of liberated sugars was measured at 488 nm and glucose (2 g/l) was used as standard solution.

3. Results

3.1. Growth and morphology of encapsulated yeast

An anaerobic cultivation using 50 ml encapsulated *S. cerevisiae* was carried out in 200 ml of defined medium with glucose as a limited nutrient. Each batch cultivation was ended when 5–10% of initial glucose concentration was still available in the medium to ensure that cells did not get into stationary phase. The duration of each cultivation batch was 4–5 h. No significant lag phase was observed throughout all batches. The biomass concentration increased linearly over the consecutive batches, and reached 23 ± 1 g/l at the end of the 20th batch cultivation. Yields of the most important metabolites on glucose and carbon recovery from different batches are summarized in Table 1.

The yield of ethanol produced was slightly increased from 0.43 to 0.46 g/g in the sequential batch cultivations. On the other hand, glycerol and biomass yields showed an overall trend to reduce over consecutive batches. Just 6.2 and 5.5% of glucose were converted to glycerol and biomass after the first batch, whereas the similar values after batch 20 were 4.8 and 2.3, respectively. This indicates a significant decrease in yield of these two by-products. Among the carboxylic acids produced, acetic, succinic and pyruvic acids were detected. The yields of these first two acids were reported in Table 1, while the yield of extracellular pyruvic acid was less than 1.0 mg/g. The yield of succinic acid was almost constant during sequential batches, while the yield of acetic acid increased slowly and was doubled at the end of batch 20 compared to the initial one, although this increase mostly occurred in the first 10 batch cultivations. As shown in Table 1, all measured metabolites accounted for 97.7 ($\pm$1%) of the consumed glucose. The specific growth rate of the yeast (μ) decreased drastically in the first 10 batch cultivations, compared to the subsequent experiments (Fig. 1a). The rate decreased from 0.13 to 0.03 (h^{-1}) over the first 10 batches, and further to 0.01 until the end of batch 20.

Samples of encapsulated yeast were collected from the capsule membranes, re-suspended and used for microscopic inspection. The numbers of cells, which were in the budding process were estimated, using Bürker counting chamber and compared to total cell numbers over the consecutive batch cultivations. The fraction of budding yeast was 91 and 85% in the cultivation batches 1 and 5, respectively, while these numbers decreased to 63 and 42% after batches 10 and 20, respectively (Fig. 1h). Many cells in the latter cultivation batch existed in the form of single and non-budding cells.

3.2. Cellular compositions of encapsulated yeast

The biomass samples obtained after mechanical rupture of capsule membranes were utilized for determination of cellular compositions. The RNA content from samples retrieved was 9.03% of the dry weight of biomass after the first batch. It steadily decreased over the sequential batches and reached around 5.5% by 20 batches (Fig. 1b). This means the average amount of total RNA as a percentage of dry weight decreased by 39% over 20 consecutive batch cultivations.

The protein content of the cells from sequential batches followed the same trend as RNA content (Fig. 1c). Protein content was approximately 46%(w/w) after the first cultivation of the encapsulated cells, while it steadily decreased to 35%(w/w) in the last cultivation, showing a 24% decrease over these 20 cultivation batches. Decrease of protein is usually accompanied by the reduction of total RNA content in the lower growth rate of yeast. However, the decrease in RNA content was more than that of the protein in the analysed samples from batches 1 to 20. Fig. 1d shows the ratio of RNA to protein for estimated samples. A ratio of 0.2 after batch 1 decreased to 0.16 after batch 10 and remained almost unchanged over the following batches.

Glycogen and trehalose are the two intracellular glucose stores of yeast cells, and accurate estimation of their concentration is essential for detailed understanding of many aspects of yeast physiology. The results for glycogen, trehalose and total carbohydrates analyses are presented in Fig. 1e,f,g, respectively. Yeast cells accumulated both the aforementioned carbohydrates and the total amount of carbohydrates as it was growing over repeated batches. The glycogen and trehalose contents at the end of the first batch were 1.96 and 0.26% of the dry weight of biomass. Both carbohydrates content increased gradually after each batch cultivation. The corresponding values in the last cultivation for glycogen and trehalose were respectively, 4.5 and 4 times higher than those from the first batch. The sum of glycogen and trehalose accounted for 2.22, 6.37 and 9.80% of the total cell mass after batches 1, 10 and 20, respectively.

Total carbohydrate measurements also showed an elevated content over sequential batches, and followed trends similar to those of the two stored carbohydrates. From the first to the 20th batch cultivations, the total carbohydrate content of yeast cells

Table 1
Yields of the most important metabolites of encapsulated *S. cerevisiae* anaerobically cultured in glucose-limited medium at various consecutive batch cultivations

Sequential batches	Y_{SE}	Y_{SGly}	Y_{SX}	Y_{SAce}	Y_{SSuc}	Y_{SC}	Carbon Recovery (%)
1	430 (±10)	62 (±3)	55 (±5)	6.0	3.0	411	96.7
4	440 (±10)	63 (±5)	45 (±4)	8.0	2.0	421	97.9
7	440 (±20)	56 (±4)	43 (±3)	8.0	2.0	421	97.0
10	450 (±20)	52 (±3)	38 (±4)	10	3.0	430	98.3
14	450 (±10)	54 (±5)	32 (±5)	11	3.0	430	98.0
20	460 (±10)	48 (±2)	23 (±3)	12	3.0	440	98.6

Y_{SE} = ethanol yield, Y_{SGly} = glycerol yield, Y_{SX} = biomass yield, Y_{SAce} = acetic acid yield, Y_{SSuc} = succinic acid yield, Y_{SC} = carbon dioxide yield. All yields were expressed as (mg/g).

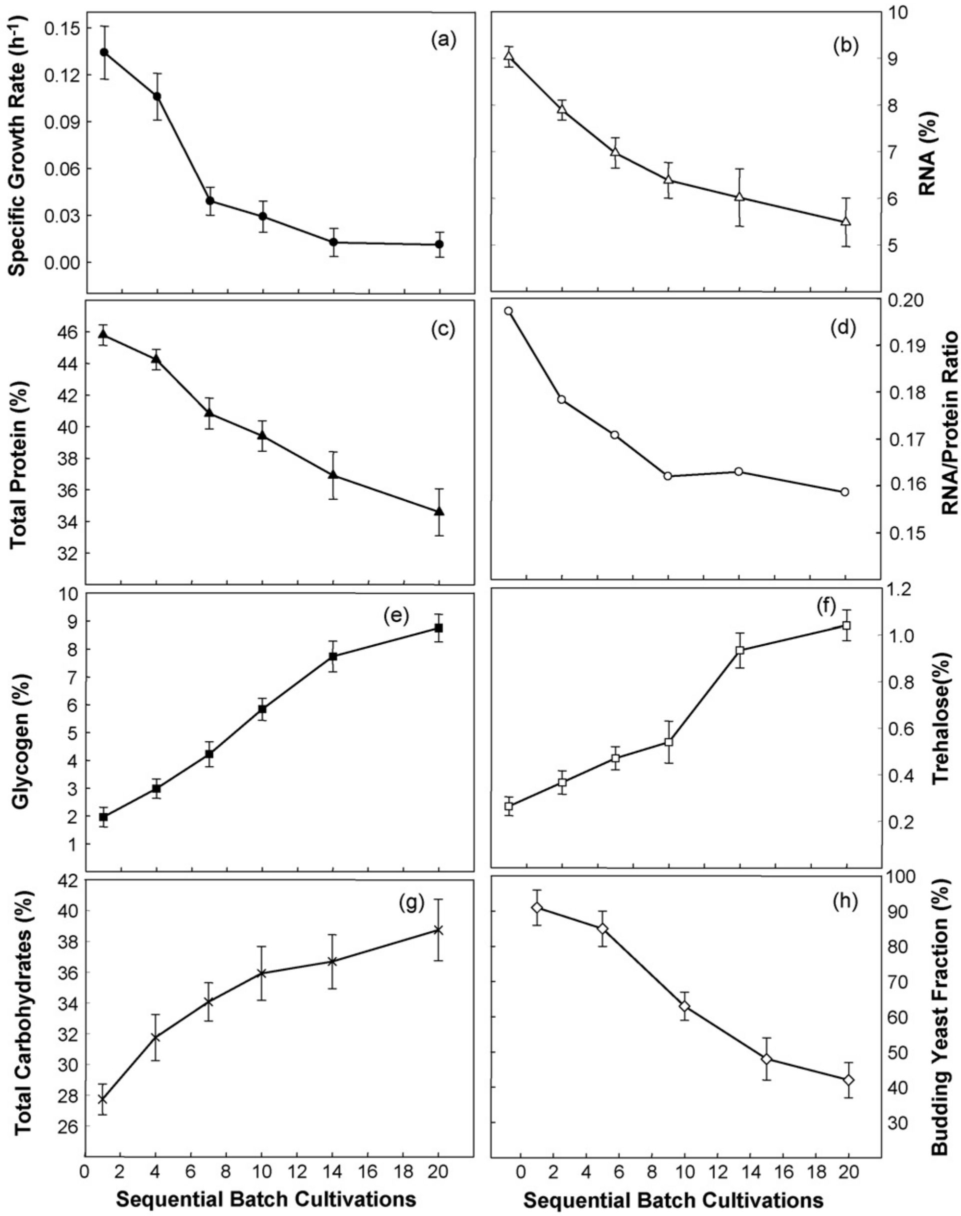

Fig. 1. Profile of (a) changes in specific growth rate (μ), (b) total RNA, (c) total protein, (d) ratio of total RNA to protein content, (e) glycogen, (f) trehalose, (g) total carbohydrates content (%w/w) of encapsulated yeast samples at different sequential batch cultivations, and (h) fraction of budding encapsulated yeast (*S. cerevisiae*) at different consecutive batches (◊) estimated on Bürker counting chamber.
Note: The results of glycogen and trehalose are expressed as the percentage of glucose equivalent per cell dry weight (%w/w).

increased from 27.5 to almost 39%, indicating 11.5% rise in carbohydrate content of the yeast biomass (Fig. 1g).

3.3. Diffusion and mass transfer coefficient over consecutive cultivations

The penetration of glucose through the capsules into their interiors was estimated and the data were applied to determine the mass transfer coefficient, based on the method described previously; the results are summarized in Fig. 2 and Table 2. As the capsules containing cells were cultivated more, the cell growth made the membrane less penetrable for glucose, leading to a lower diffusion rate. The value of the volumetric mass transfer coefficient estimated for glucose penetrating through the membrane of cell-free capsules was 6.28 (cm^3/min) while corresponding values for capsules containing cells showed a decreasing trend as the capsules were exposed to more batch cultivations. After the 20th batch cultivation, when the concen-

Table 2
Calculated volumetric mass transfer coefficient (K) of glucose into empty capsules and those collected from different batches of the sequential cultivations with encapsulated cells

Samples	K (cm^3/min)
Cell-free capsules	6.28
Capsules from the 5th batch	4.11
Capsules from the 10th batch	3.70
Capsules from the 20th batch	1.24

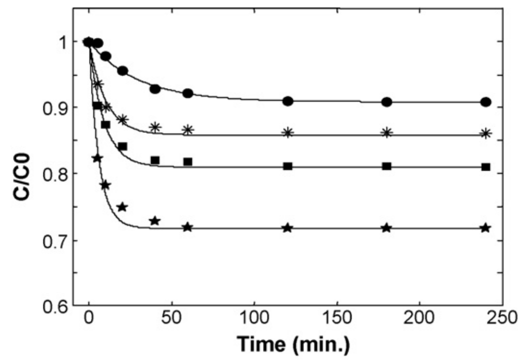

Fig. 2. Time course of glucose diffusion through the cell-free (⋆) and cell-seeded capsules membrane after 5 (■), 10 (*) and 20 (●) batch cultivations. The lines show the profile of diffusion according to Eq. (4).

tration of yeast cells inside the capsules was very high, the mass transfer coefficient had a value of 1.24 (cm^3/min), which was only 20% of its initial value for cell-free capsules (Table 2).

4. Discussion

Alteration in metabolism of encapsulated yeast has not previously been studied. Encapsulation is regarded as one of the most effective techniques for cell retention, which has been shown to have many advantages compared to the other conventional immobilization techniques [21]. Altered metabolism in immobilized cells has been evidenced in several investigations [12,13,15], but the cause of these changes is not yet fully elucidated, and is often attributed to modification of the microenvironment around the immobilized cells, or to intrinsic alteration of cellular physiology induced by immobilization [14,15]. The current results demonstrate more details on the growth pattern of encapsulated cells, the physiological and morphological changes of cells under encapsulation, and their fermentation capacity.

The volumetric mass transfer coefficient is a variable influenced by both variables k and a. If the cell growth in the capsules' interior occurs homogeneously, then it might lead to a reduced value of k by progressing with the experiment. The observed growth pattern of encapsulated yeast indicates that the cells are not uniformly distributed inside the capsules; instead they tend to attach to the membrane and grow from one side, which may suggest that the main variable affected is a. As the size of the cell community increases, the effective surface area through which glucose can diffuse decreases, leading to a reduced K value. Reduction in K value, therefore, can mainly be attributed to the decrease in effective surface area of capsules, with the assumption that the diffusion rate into the packed-cell community is negligible compared to that through the membrane. Decrease in specific growth rate of encapsulated cells was in agreement with the reduced fraction of budding yeast over the cultivation sequences (Fig. 1a,h). Both glucose limitation due to the lower rate of mass transfer (Fig. 2) and cell attachment to the membrane and/or other cells might be responsible for inhibition of budding in encapsulated cells in the inner layer of the cell community [12].

Continuous cultivation of *S. cerevisiae* CBS 8066 in glucose-limited medium [22], showed that decreasing dilution rate – which is equal to μ – resulted in decreasing protein and RNA content, while stored and total carbohydrate increased at the same time (Table 3). This trend is similar to what we obtained from the encapsulated yeast. The protein content of the freely suspended cells at dilution rate (and even μ at) 0.10 h^{-1} is equal to our 4th batch cultivation with encapsulated yeast, where we measured μ at 0.10 h^{-1} (Table 3). On the other hand, comparison of the RNA/protein ratio in these two conditions shows 28% higher value within the encapsulated cells than in the freely suspended cells, while glycogen, trehalose and total carbohydrates content of encapsulated cells were 35, 45 and 68% of those in freely suspended cells, respectively (Table 3). This might be an indication of a deeper difference between free and encapsulated cells than we can interpret with just the growth rate factor.

The decrease in total RNA, protein content and RNA/protein ratio due to growth impairment in various organisms such as bacteria and yeast has been reported (e.g. cf. Refs. [23,24]). A linear proportionality between the specific growth rate and the RNA/protein ratio for freely-suspended cells has been reported and thus, the RNA/protein ratio could be a good indication of cell growth rate in different media or conditions [25]. The results of current work indicate that a decreased specific growth rate is accompanied by a decrease in RNA/protein ratio (Fig. 1a,d), but these two variables for encapsulated cells are not proportionally decreased which differ from freely-suspended cells.

Glycogen and trehalose are two reserved carbohydrates accumulated under slow growth of microbial cells due to nutrient limitation. Trehalose is also accumulated in cells exposed to environmental stress. The role of trehalose, in addition to being a pool of glucose storage in the cells, is to protect cytosol com-

Table 3
Comparison of changes in biochemical cellular composition of *S. cerevisiae* CBS 8066 in encapsulated (current work) and free cell [22] growth with respect to different growth rates

Specific growth rate and cellular content (%)	Free cells in continuous cultivation		Encapsulated cells in sequential batch cultivations		
	$D = 0.4 (h^{-1})$	$D = 0.1 (h^{-1})$	Batch 1	Batch 4	Batch 20
μ (h^{-1})	0.40	0.10	0.13	0.10	0.01
Proteins	60.1	45	45.8	44.2	34.6
RNA	12.1	6.3	9.13	7.89	5.85
Glycogen	0	8.4	1.96	2.98	8.75
Trehalose	0	0.8	0.26	0.36	1.04
Carbohydrates	17	40.7	27.7	31.7	38.9

ponents against adverse growth conditions such as heat and osmotic shock or nutrient depletion [26]. The prolonged growth of the encapsulated cells resulted in higher accumulation of these carbohydrates (Fig. 1e,f). There is parity in increase of these carbohydrates and the total carbohydrates (Fig. 1e,f,g, Table 3). It might indicate that glycogen and trehalose are the major components of the total carbohydrates that are varied in prolonged cultivation of encapsulated cells, while the structural carbohydrates in the cell wall, that is, glucan and mannan, are not or less affected by encapsulation. This is reasonable since the growth rate proved to have little effect on structural carbohydrates. Increase in the average level of glycogen and trehalose could indeed be attributed to the yeast cells inside the packed community, whose growth has been suppressed due to attachment and/or lack of nutrients, since the cells accumulate the stored carbohydrates under such conditions [12]. This unique growth pattern of cells with altered cell compositions and perhaps appearance of new proteins as reported by Perrot et al. [20] could play a role in giving higher resistance to encapsulated yeast to survive and grow in highly toxic medium [9].

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Paper V

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Full Research Paper

Protective Effect of Encapsulation in Fermentation of Limonene-contained Media and Orange Peel Hydrolyzate

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Abstract: This work deals with the application of encapsulation technology to eliminate inhibition by D-limonene in fermentation of orange wastes to ethanol. Orange peel was enzymatically hydrolyzed with cellulase and pectinase. However, fermentation of the released sugars in this hydrolyzate by freely suspended *S. cerevisiae* failed due to inhibition by limonene. On the other hand, encapsulation of *S. cerevisiae* in alginate membranes was a powerful tool to overcome the negative effects of limonene. The encapsulated cells were able to ferment the orange peel hydrolyzate in 7 h, and produce ethanol with a yield of 0.44 g/g fermentable sugars. Cultivation of the encapsulated yeast in defined medium was successful, even in the presence of 1.5% (v/v) limonene. The capsules' membranes were selectively permeable to the sugars and the other nutrients, but not limonene. While 1% (v/v) limonene was present in the culture, its concentration inside the capsules was not more than 0.054% (v/v).

Keywords: Orange peel, encapsulated yeast, alginate membrane, ethanol, limonene, enzymatic hydrolysis

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1. Introduction

The orange is one of the major citrus fruits and its production has increased since the 1980s. Orange production is predicted to approach 66.4 million tons by 2010, representing a 14% increase within 12 years [1]. Approximately 40-60% of oranges are squeezed to juice and the remainder, containing peel, segment membranes and other by-products is considered as citrus processing waste (hereafter referred to as orange peel for simplicity) [2]. Part of this waste is dried to be used as animal feed, but the drying process is costly due to the high moisture content of peels, and therefore a large proportion of waste has to be disposed of. This may result in many problems from both economic and environmental points of view including high transport costs, lack of disposal sites and high organic content [3]. Citrus processing residues contain both soluble and insoluble carbohydrates. The latter are present in the cell walls of the peels, particularly in the form of pectin, cellulose and hemicellulose. These polymers can be hydrolyzed enzymatically by cellulase, β-glucosidase and pectinase to their corresponding soluble carbohydrates [2,4].

Production of ethanol and other valuable products from fermentable sugars in peel hydrolyzate is an alternative to utilize industrial citrus processing waste and avoids the disposal-associated problems. However, the main obstacle to fermentation of orange peel is the presence of peel oil (more than 95% D-limonene, hereafter called limonene), a component that is extremely toxic to fermenting microorganisms. The antimicrobial effect of limonene was reported even at very low concentrations such as 0.01% (w/v), and resulted in complete failure of fermentations at higher concentrations [5-7]. Therefore, a successful fermentation usually requires prior separation of limonene from the medium, by e.g. filtration or aeration [7,8]. Enclosing of yeast cells inside a selective and porous membrane through encapsulation could be a solution when toxic media are to be fermented. In the encapsulation method, a membrane is designed in which the nutrients and products can easily pass through while the toxic materials are excluded [9]. Encapsulation has already been presented as a powerful tool in fermentation of toxic lignocellulosic hydrolyzates, where strong inhibitors such as furfural were present in the culture [10].

The current work deals with the development of the encapsulation technique in fermentation of orange peel hydrolyzates in the presence of limonene, even at very high concentrations. In this method, yeast cells are confined within a hydrophilic membrane, which is theoretically impermeable to components with a hydrophobic nature like limonene [10]. The function of this membrane is to act as a selective barrier that protects the yeast cells and prevents them from being in direct contact with the toxic oil. The performance of encapsulated *S. cerevisiae* in both synthetic media containing high concentration of limonene and orange peel hydrolyzate and the membrane's selectivity were particularly investigated.

2. Results and Discussion

2.1. Enzymatic Hydrolysis

Hydrolysis of Argentina orange peel was carried out in both bioreactor and shake flask at 45 ˚C for 24 h with 12% solid concentration. The dry matter content of the peels was 20% (± 1.2%). However, the concentrations of more than 12% resulted in practical difficulties for efficient mixing in the bioreactor. The respective loadings of pectinase, cellulase and β-glucosidase were 1163 IU/g, 0.24 FPU/g and 3.9 IU/g peel dry matter, based on optimized values previously reported for grapefruit peel [4]. The yields of sugars liberated after the hydrolysis are summarized in Table 1. There was no significant difference between the results from shake flask and the bioreactor. The released materials during the enzymatic hydrolysis were glucose, fructose, galactose, arabinose, xylose and galacturonic acid (GA).

Table 1. Yields of the carbohydrates released during enzymatic hydrolysis of the orange peels.

Carbohydrate	% (of total solid)
Glucose	22.9 ± 2.4
Fructose	14.1 ± 1.3
Galactose	4.0 ± 0.2
Arabinose	7.1 ± 0.5
Xylose	0.4 ± 0.1
Galacturonic acid	19.0 ± 1.7
Total	67.5

The sum of these sugars and sugar acid in hydrolyzate accounts for 67.5% of total solid content of the peels, where glucose, fructose and GA are the main three components present in hydrolyzate, corresponding to 84% of total sugars and sugar acid. The results obtained in the current work are similar to those reported previously by other researchers [4,7]. Concentration of limonene in the hydrolyzate was 0.52% (v/v), which can be compared to limonene concentration of 1.4% (v/v) for Valencia peel hydrolyzate with 23% initial dry matter [7] .

2.2. Free cell cultivation

Anaerobic batch cultivations of “freely suspended” *S. cerevisiae* were carried out in synthetic medium containing 0, 0.5, 1 and 1.5% (v/v) of limonene, and the most important results are presented in Figures 1-2 and Table 2. The initial cell concentration in each flask was 3.6 (± 0.3) g/L (approximately 10^7 cells/mL). The suspended cells were not able to assimilate glucose in any of the media containing limonene at different concentrations. However, in the absence of limonene, the suspended cells were able to assimilate glucose completely in 12 h and produced ethanol with yield 0.41 (± 0.03) g/g (Figures 1a,b and Table 2).

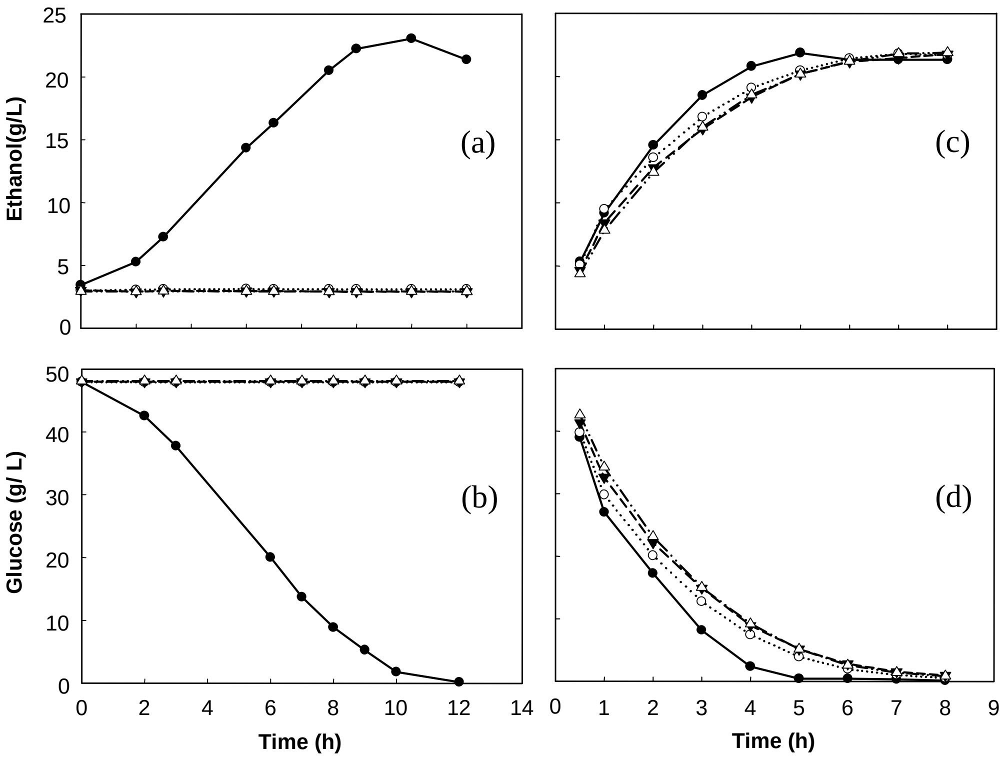

Figure 1. Glucose and ethanol concentration in anaerobic batch cultivation with suspended (a,b) and encapsulated *S. cerevisiae* (c,d), where limonene with different concentrations of (v/v) 0% (●), 0.5% (○), 1%(▼), 1.5% (∆) was present in the culture.

The fraction of viable cells was estimated by the colony forming units' method (CFU) at different concentrations of limonene for suspended-cell system and is depicted in Figure 2.

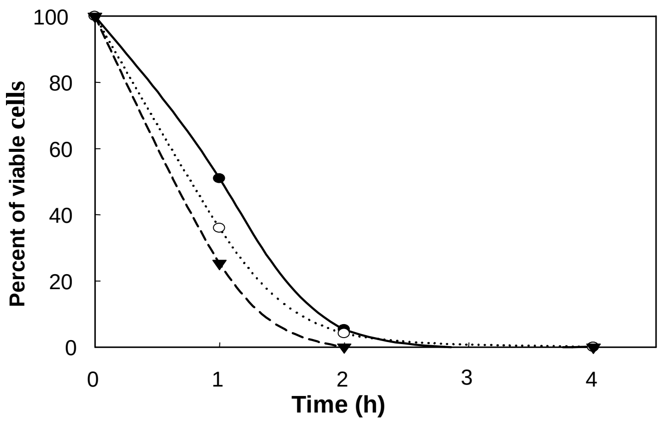

Figure 2. Effect of 0.5% (●), 1% (○), 1.5% (▼) limonene on viability of *S. cerevisiae* in batch cultivation with suspended cells.

The fraction of viable cells in defined media containing 0.5, 1 and 1.5% (v/v) of limonene decreased to 51, 36 and 25% of the initial values, respectively, within 1 h cultivation. Furthermore, the number of viable cells for all media containing limonene was practically zero after 4 h cultivation. It indicates a high level of toxicity of limonene towards the strain of *S. cerevisiae* used.

2.3. Cultivation of encapsulated cells in the presence of limonene

Anaerobic batch cultivations of encapsulated *S. cerevisiae* were carried out in synthetic medium containing 0, 0.5, 1 and 1.5% (v/v) limonene. The most important results are presented in Figures 1c,d and Table 2. A total volume of 25 mL encapsulated cells (825 ± 25 capsules) in 100 mL defined medium was used, where 3.6 (± 0.3) g/L of the cells were initially present in the capsules. It was similar in cultivation conditions to the suspended cells, in which the cells failed to assimilate glucose in any of the synthetic media containing limonene at different concentrations. However, cultivation of encapsulated cells was successful and glucose was assimilated even at very high limonene concentration of 1.5% (v/v) (Figures 1c,d and Table 2). Cultivation in the limonene-free medium was completed in 5 h, while the cultivations in the presence of 0.5 to 1.5% (v/v) limonene ended in 7 h. On the other hand, increasing the limonene concentration in this range had practically no effect on fermentation time.

Table 2. Key rates and yields in anaerobic batch cultivation of suspended cells in limonene-free medium and encapsulated cells in media with various concentrations of limonene and peel hydrolyzates.

Parameter	Suspended Cells	Encapsulated cells with different limonene concentration (% v/v) of:				Peel hydrolyzate
		0	**0.5**	**1**	**1.5**	
$-r_S$ (g/L.h)	4.0	8.56	5.96	5.38	5.58	5.00
r_E(g/L.h)	1.6	3.69	2.57	2.28	2.33	2.18
Y_{SE}(g/g)	0.41(±0.03)	0.43(±0.02)	0.43(±0.01)	0.42(±0.01)	0.42(±0.01)	0.44(±0.01)
Y_{SGly}(g/g)	0.080	0.062	0.050	0.048	0.047	0.050
Y_{SAce}(g/g)	0.018	0.017	0.016	0.016	0.015	0.017
Y_{SSuc}(g/g)	0.003	0.003	0.003	0.003	0.003	0.003

*Notes: $-r_S$, volumetric glucose uptake rate; r_E, volumetric ethanol production rate; Y_{SE}, ethanol yield; Y_{SAce}, acetic acid yield; Y_{SSuc}, succinic acid yield; Y_{SGly}, glycerol yield

Ethanol production and glucose uptake rate were affected by the presence of limonene. Ethanol productivity in the media containing 0, 0.5, 1, and 1.5% (v/v) limonene was 3.69, 2.57, 2.28 and 2.33 g/L.h, respectively. It shows a clear difference between the presence and absence of limonene in the culture. However, the effect of increasing limonene concentration by threefold from 0.5 to 1.5% (v/v) on the ethanol productivity might be in the range of experimental variation. On the other hand, ethanol productivity of the encapsulated cells in the presence of limonene was still higher than the corresponding value with the suspended cells in limonene-free medium (cf. Table 2).

Yield of ethanol produced was not significantly affected by the presence of limonene in the synthetic media and remained constant at 0.425 (± 0.005) g/g. Glycerol yield, on the other hand, was slightly decreased from 0.062 to 0.050 g/g by the presence of 0.5% limonene. Further increase in limonene concentration, however, had a minor effect on reduction of glycerol yield. In general, glycerol yield was lower for encapsulated yeast cultivation compared to the corresponding value of 0.080 g/g for suspended-cell cultivation. Acetic and succinic acids were also produced in all the cultivations, but in trace amounts, and no significant effect of limonene on their yield was observed (Table 2).

2.4. Yeast cultivation in peel hydrolyzate

Orange peels that were enzymatically hydrolyzed were supplemented with nutrients and anaerobically cultivated by both freely suspended and encapsulated *S. cerevisiae* in the bioreactor with total working volume of 1 L at 30 ˚C. The hydrolyzate contained limonene whose concentration was measured as 0.52% (v/v). The suspended cells were not able to ferment the peel hydrolyzate in 24 h, where no sugars could be taken up by *S. cerevisiae* and no ethanol was produced.

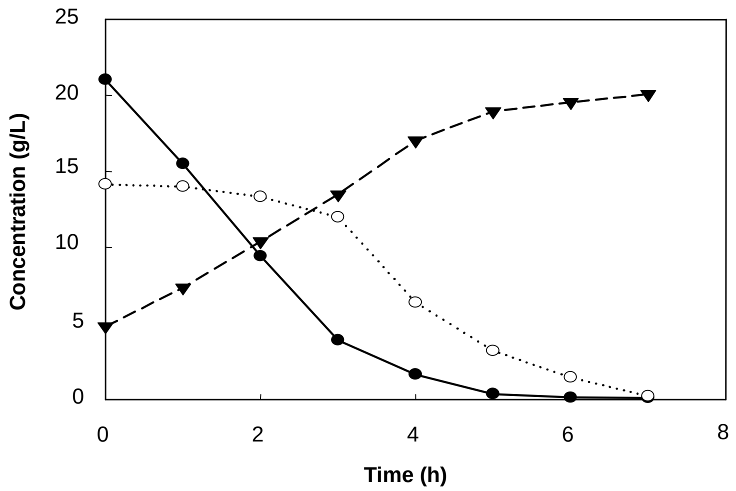

Figure 3. Profiles of glucose (●), fructose (○) and ethanol (▼) in cultivation of orange peel hydrolyzate by encapsulated *S. cerevisiae*.

Encapsulated *S. cerevisiae* successfully converted the fermentable sugars to ethanol and other metabolites. Among the sugars available in peel hydrolyzate, only glucose and fructose could be assimilated by the applied yeast strain and fermentation was completed within 7 h. Consumption of fructose was delayed by the presence of glucose and the yeast started to take up fructose after the concentration of glucose approached below 5 g/L (Figure 3). Ethanol yield based on total sugar consumption was 0.44 (± 0.01) g/g, and yields of glycerol, acetic and succinic acids were similar to the corresponding values of 0.5% (v/v) limonene in synthetic medium (Table 2).

2.5. Selectivity of capsules' membrane:

The liquid content of 350 capsules after crushing was used for determination of limonene by GC-MS and the result showed a concentration of 0.054% (v/v) of this toxic material inside the capsules. This result was also confirmed by the CFU test (Figure 4) where about 10^5 cells/mL were found viable after 24 h incubation. It means that the viability of the encapsulated cells at this condition was stable at 8.5% (± 1.5%) within 4-24 h cultivation. This number is comparable to the viability of suspended cell at limonene concentration of 0.02 and 0.07% (v/v) in the media.

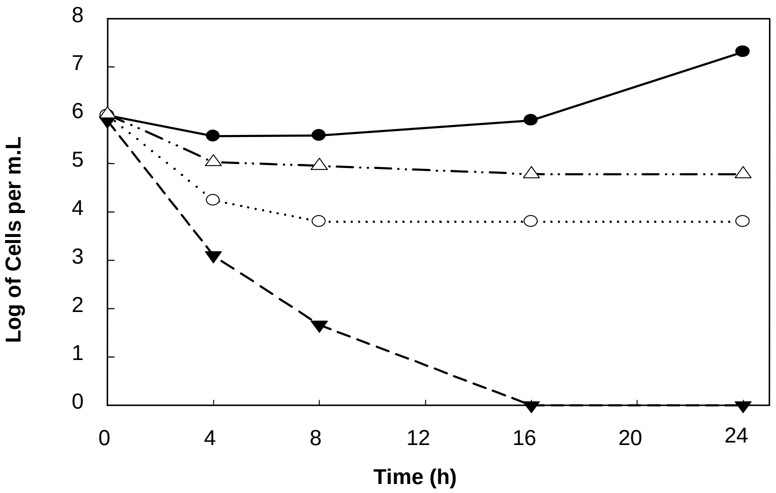

Figure 4. Effect of 0.02% (●), 0.07% (○), 0.1% (▼) (v/v) limonene on viability of suspended *S. cerevisiae* compared to 0.054% limonene (Δ) on the encapsulated yeast.

These results indicate that the suspended cells at initial concentration 10^6 cells/mL could tolerate the presence of 0.02% (v/v) limonene in the medium even though the number of viable cells was slightly decreased in the first few hours and the growth of cells was delayed for 16 h (Figure 4). Media with 0.07 and 0.1% (v/v) of limonene were extremely harsh for *S. cerevisiae*, in which 98.3 and 99.8% of cells were not viable after 4 h, respectively (Figure 4).

2.6. Conclusions:

Fermentation of orange peel hydrolyzate obtained from enzymatic hydrolysis in various solid concentrations was previously reported by freely suspended cells. However, fermentation was successful only at the lower solid concentration (2%), while pretreatment to remove the existing limonene was reported as a demand at the higher solid concentration [7]. The lethal concentration of limonene is as low as 0.1% (v/v). It is even a strong inhibitor at lower concentrations where the cells' viability is decreased within a few hours and the yeast population extensively decreases to a constant level (Figure 4). Yeasts are usually more sensitive to limonene than bacteria [15,16] and minimum inhibitory concentration of limonene at 0.01% (w/v) has been reported for *S. cerevisiae* [6]. However, this value may change depending on the medium pH [5]. Limonene is a hydrophobic component that can pass freely through the cell wall of yeast [17] and inhibit lipid body formation and accumulation inside the cell [18].

Preventing the cells from being in direct contact with limonene could be an efficient way to protect them from harsh environmental conditions such as organic solvent and poison. Encapsulation through which microbial cells are confined in a semi-permeable membrane is considered to be a promising method for cell retention but also for cell protection [9,19]. Due to the hydrophobic nature of limonene, it can be expected that alginate capsules' membrane would not be permeable to limonene, and fermentation should be possible regardless of the presence of limonene in the medium even at high concentration. If the capsules' membrane functions as an ideal selective barrier in which no passage of limonene occurs, then addition of limonene should not have any impact on cultivation with encapsulated cells. However, the results indicated that the fermentation rate and metabolite production were influenced by the presence of limonene in the media regardless of its concentration (Table 2). This fact suggests that limonene partially passes through the membrane and consequently can affect encapsulated yeast.

In conclusion, limonene has an enormous lethal effect on *S. cerevisiae 8066*. This fact indicates that practically no peel hydrolyzate can be fermented to ethanol without pretreatment. Application of encapsulated yeast, however, makes it possible to ferment peel hydrolyzate even with high limonene content. Although a minor amount of limonene penetrated into the capsules' interior and showed inhibitory effects, the yeast colonies inside the capsules were able to completely assimilate the sugars within 7 h even in a medium containing 1.5% (v/v) limonene.

3. Material and Methods

3.1. Substrates and enzymes

The peels were residuals from Argentina orange obtained from Brämhults juice factory (Borås, Sweden) and stored frozen at -20 °C until use. The frozen peel was thawed and ground with a food homogenizer (ULTRA-TURAX, TP 18-20, Janke & Kunkel Ika-Labortechnik, Germany) to less than 2 mm in diameter. Total dry content of orange peel was determined by drying at 70 °C for 48 h. Three commercial enzymes, Pectinase (Pectinex Ultra SP), Cellulase (Celluclast 1.5 L) and β-glucosidase (Novozym 188), were provided by Novozymes A/S (Bagsvaerd, Denmark). Pectinase activity was measured by hydrolyzing 0.02% citrus pectin solution at 45 °C in 50 mM sodium acetate buffer at pH 4.8 [4], and it was 283 international units (IU)/mg protein. Cellulase activity was determined based on a standard method provided by National Renewable Energy Laboratory [11], and was 0.12 filter paper units (FPU)/mg protein. Activity of β-glucosidase was 2.6 IU/mg solid as reported by the supplier.

3.2. Hydrolyses

Enzymatic hydrolyses of the peels were carried out in both shake flasks and a bioreactor (Biostat A., B. Braun Biotech, Germany). The experiment in shake flasks was performed to measure the concentration of sugars and limonene. In this case, ground peel was added into 250 mL conical flasks containing 50 mM sodium acetate buffer at pH 4.8 to make 100 mL of peel/water slurry with a solids concentration of 12%. The flasks were then placed in a shaker bath at 45 °C and 140 rpm for 24 h. Higher volumes of hydrolyzates were prepared by hydrolysis of ground peel in the bioreactor with a working volume of 2 L and 12% solid concentration at 45 °C with stirring rate of 500 rpm for 24 h. The pH of the slurry was controlled at 4.8 by addition of 2 M NaOH.

3.3. Yeast Strain and Media

The yeast *S. cerevisiae CBS 8066*, obtained from Centraalbureau voor Schimmelcultures (Delft, The Netherlands) was used in all experiments. The strain was maintained on agar plates made from yeast extract (10 g/L), soy peptone (20 g/L), and agar (20 g/L) with D-glucose (20 g/L) as an additional carbon source. The media used were either defined synthetic medium [12] containing glucose (50 g/L) and 0, 0.5, 1 and 1.5% (v/v) of limonene (89188, Fluka, UK), or orange peel hydrolyzates supplemented with appropriate amounts of all the mineral salts and the trace elements to make the same composition as in synthetic medium.

3.4. Encapsulation Method and Batch Cultivation Experiments

The cells of *S. cerevisiae* were encapsulated using alginate-calcium membrane developed by Talebnia *et al*. [10], where yeast cells (3.6 g/L) were present in the capsules at the beginning of the experiments. Furthermore, cultures of 3.6 g/L freely suspended cells were provided for comparison with the encapsulated cells. These encapsulated and freely suspended cells were anaerobically cultivated in 100 mL defined medium containing 0, 0.5, 1 and 1.5% (v/v) limonene under identical conditions in 250 mL conical flasks. The flasks equipped with two stainless steel capillaries, and a glass tube with a loop trap, were used on the shaker bath at 30 °C and 140 rpm for this set of experiments [13]. Fermentation of orange peel hydrolyzate was carried out in the bioreactor where temperature, stirring rate and pH were controlled at 30 °C, 200 rpm and 5, respectively. A total volume of 200 mL cell-seeded capsules after 16 h aerobic cultivation was added to bioreactor containing 800 mL hydrolyzate. Nitrogen gas was steadily sparged at the rate of 600 mL/min.

3.5. Selectivity of capsules' membrane

In order to characterize the selectivity of membrane, both direct and indirect methods were applied. The interior content of 350 capsules after mechanical rupture was utilized for GC-MS analysis where its oil content was previously extracted in *n*-heptane (12 mL). Through the indirect method, 25 mL of cell culture were cultivated in 100 mL synthetic media containing 0.02, 0.07 and 0.1% (v/v) of limonene to make an initial concentration of 10^6 cells/mL. Then, the viability of cells was estimated with the colony forming units' method (CFU). A similar test was carried out where 25 mL of cell culture was encapsulated and added to 100 mL synthetic medium containing 1% (v/v) limonene and the same initial cell concentration (10^6 cells/min).

3.6. Analytical methods

An Aminex HPX-87P ion-exchange column (Bio-Rad, USA) was used at 85 °C for estimation of glucose, galactose, arabinose, xylose and fructose concentrations. Ultra-pure water was used as eluent at a flow rate of 0.6 mL/min. Ethanol, acetic, galacturonic, succinic and pyruvic acids and glycerol concentrations were determined on an Aminex HPX-87H column (Bio-Rad, USA) at 60 °C using 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. A refractive index (RI) detector (Waters 2414, Milipore, Milford, USA) and UV absorbance detector at 210 nm (Waters 2487) were used in series. Succinic acid was analyzed from UV chromatograms while the rest of metabolites were quantified from the RI chromatograms.

The cell viability and cell dry weight were determined according to Talebnia *et al*. [14]. The concentration of limonene was determined by addition of *n*-heptane (40 ml, 99% purity) to the hydrolyzate (100 ml) and centrifugation at 3500 g for 30 min to extract the oil. The resulting supernatant was then analyzed by a GC-MS (Hewlett Packard G1800C, Agilent, Palo Alto, CA) where the carrier gas was helium. The temperature was initially 50 °C and was increased to 250 °C at the rate of 15 °C/min and maintained at this temperature for 3 min.

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