Report for the evaluation of the Swedish Energy Agency´s research programme Ethanol from cellulose 2007-2010

Project:

Ethanol production from lignocelluloses by encapsulated and flocculating yeast

Project number:
P13872-2

Project period:
2009-01-01 – 2010-12-31

Project leader
Mohammad Taherzadeh
School of Engineering
University of Borås, 501 90, Borås
Tel: 033-435 5908, Fax: 033-435 4008
E-mail: Mohammad.Taherzadeh@hb.se

Summary

Cultivation with high-cell density can decrease the fermentation time as well as increasing the tolerance of the cells against the inhibitors. Cell recycling by e.g. centrifuges is a method to increase cell concentration inside the bioreactors. This method has been widely used in industries for ethanol production from e.g. wheat in different processes such as the Swedish Biostill®. However, the toxicity of the lignocellulosic hydrolyzates and presence of small particles in the hydrolyzate have not allowed this method yet to be well-developed. Furthermore, high shear stress caused by centrifugation may significantly damage the cells, and decreases their viability. On the other hand, this centrifugation can be simplified or even avoided using flocculating yeast. Flocculating yeast cells have been widely investigated and used for separating yeast cells from beer in the brewing industry. A lectin-like protein – called flocculins – stick out of the cell walls and selectively bind mannose residues present in adjacent yeast cells. This flocculating process is a reversible, asexual and calcium-dependent process. Since the flocculated cells can easily be separated in a simple settler, less mechanical stress and high cell viability can be maintained.

Immobilization of the yeast cells is another method of cell retention inside the bioreactors. A drawback of the conventional cell entrapment methods using e.g. alginate beads, is that the cells in large porous beads grow only in the periphery of the bead because of substrate and oxygen limitation. The cells may also easily leak from the gel matrix and grow in the medium. A different method of immobilization, “cell encapsulation”, is a very promising method which has many advantages over the conventional immobilizing methods. During encapsulation, cells are confined in a semipermeable, spherical and thin membrane. Due to the small wall thickness and the absence of a solid or gelled core, the mass transfer resistance is less than that of the entrapped systems. The membrane of the capsules should preferably be designed such that the required nutrients and cell products can pass through it easily, while cells and inhibitory compounds cannot. Encapsulation can be carried out by using either natural or synthetic polymers such as calcium-alginate, carrageenan-oligochitosan, chitosan-CMC, alginate-poly L-lysine, alginate-oligochitosan, alginate-aminopropylsilicate, agarose, and polyamide.

Goals of the project

The current project aimed to investigate and further improve these performance factors, and to enable conversion of lab scale results into a robust industrial process. This would be achieved by:

a) Improving the encapsulation procedures to achieve long-term robust cultivation of spent sulfite liquor and undetoxified dilute-acid lignocellulose hydrolyzates at minimum cost for the encapsulation process,

b) Clarifying the cellular mechanisms that lead to the improved yields, production rates and sugar conversion, by a detailed study of the physiology of encapsulated and the isolated flocculating yeast from the ethanol plant at Domsjö Fabriker AB,

c) Computer simulation of the entire ethanol process from lignocellulosic materials using encapsulated and flocculating yeast by ASPEN Plus, and performing an economical evaluation and optimization of the process by minimizing the energy used.

Results obtained

Improving the encapsulation procedures

Our work on natural capsules has mainly focused on using crosslinking agents (e.g. glutaraldehyde) to introduce covalent bonds to the Ca-alginate and chitosan-alginate matrix. By treating chitosan covered alginate capsules with 1% glutaraldehyde solution at pH 3 the capsules became harder and less elastic. The treatment was performed in shake flasks in a water bath. After the treatment, the capsules had changed color from white-transparent to yellow due to the crosslinking. However, an important drawback of this method has been the toxicity of glutaraldehyde for the yeasts. This toxicity was therefore investigated by adding different amounts of glutaraldehyde (0.25% to 0.0025%) into yeast containing medium. The cells were viable only in cultivations using 0.0025% of glutaraldehyde. This work needs further investigations, but already presented in a couple of conferences.

New synthetic membranes for the encapsulation procedures

Since the alginate-based membranes were not quite stable, we decided to focus more on synthetic membranes. The first membranes for this purpose was produced from polyamide 4.6 via dissolving polyamide in formic acid and PVP in ratio 12, 100, 5 and mixing for 4 h, then poured in glass casts for air casting drying. Then, encapsulation of *Saccharomyces cerevisiae* by these welded membranes were carried out. It was then continued by modification of nylon 4.6 for more hydrophilicity for making negative charge polyamide 4.6. For this purpose, we hydrophilized the nylon with an aldehyde and the membranes were produced as mentioned above (Fig. 1). The membranes were then used for encapsulation of the yeast cells and fermentation. A manuscript on these membranes is under development.

Fig. 1. Encapsulation of baker's yeast in alginate-chitosan (left-side) and synthetic membranes (right side)

A further development of this system is being carried out by developing a membrane-bioreactor system. The reactor is similar to distillation columns, in which microorganisms are located between two flat membranes and the liquid can pass through the membranes (Fig. 2). The initial experiments on this system look promising, but the system needs further development.

Higher temperature tolerace of encapsulated cells

The possibility of performing fermentations at higher temperatures would improve the enzymatic hydrolysis in simultaneous saccharification and fermentation (SSF). Therefore, the ability of encapsulated *S. cerevisiae* CBS 8066 to produce ethanol at elevated temperatures was investigated in consecutive batch and continuous cultures. Prior to the cultivation, yeast was confined inside alginate-chitosan capsules composed of an outer semi-permeable

membrane and an inner liquid core. While running one batch, the encapsulated cells were able to assimilate sugars and produce ethanol even until 50ºC (Fig. 1). However, in sequential batches, this ability was reduced. In the following experiments, the encapsulated yeast could successfully ferment 30 g/l glucose and produce ethanol at a high yield in five consecutive batches of 12 h duration at 42 °C, while freely suspended yeast was completely inactive already in the third batch. A high ethanol production was then obtained through the first 48 h at 40 °C during continuous cultivation at D 0.2 h^{-1} when using encapsulated cells. The ethanol production slowly decreased in the following days at 40°C. The ethanol production was also measured in a continuous cultivation where the temperature was periodically increased to 42-45 °C and lowered to 37°C again during periods of 12 h. In the continuous cultivation with periodic temperatures ethanol production was steady during 12 h periods at both 42 °C and 43°C, while it decreased rapidly at 44 °C and 45 °C. However, when the temperature was lowered to 37 °C the yeast cells recovered and the ethanol production increased to normal levels again. Our results show that a non-thermotolerant yeast strain improved its heat tolerance upon encapsulation, and could produce ethanol at temperatures as high as 45 °C for a short time. This work is in manuscript form at the moment.

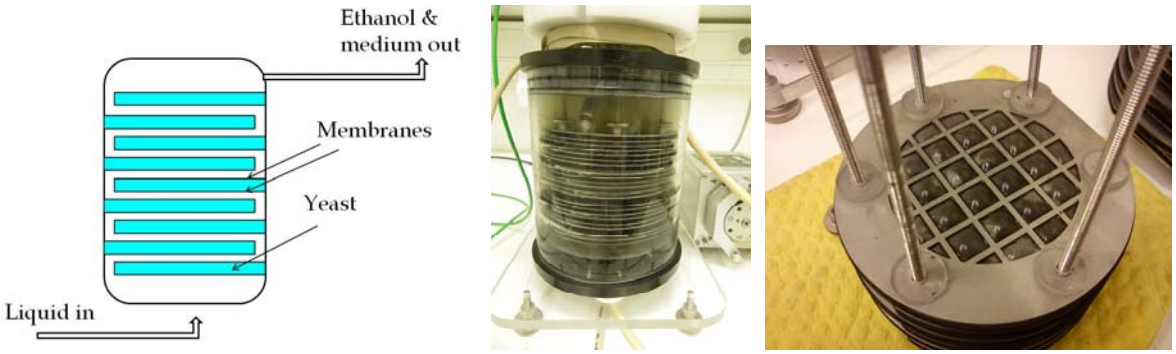

Fig. 2. A membrane-bioreactor for using synthetic membranes for ethanol production.

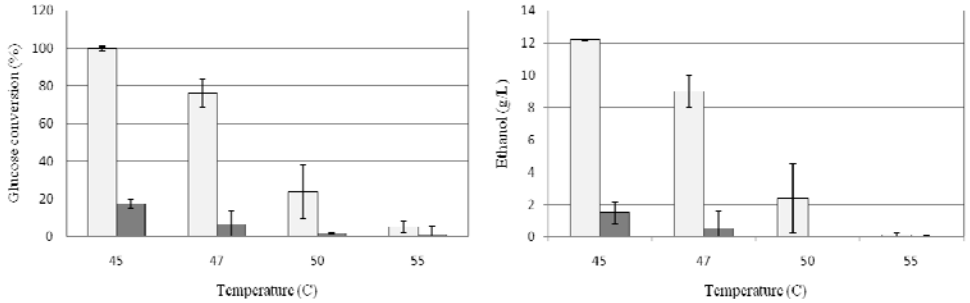

Fig. 1: Glucose consumption (left) and ethanol production (right) after 24 h anaerobic cultivation of encapsulated (white bars) and suspended (gray bars) *S. cerevisiae*.

Flocculating yeast

In this work, a constitutively flocculating strain (CCUG 53310), isolated from the ethanol plant Domsjö Fabriker AB was used. This strain has shown to be able to ferment toxic dilute acid hydrolyzates, produced at SEKAB. We tried to characterize its flocculation mechanism. The results indicated that its flocculation depended on cell wall proteins, Ca^{2+} and was inhibited by mannose. Q-PCR analysis indicated that flocculation in CCUG 53310 was mediated by expression of FLO8, leading to expression of FLO10 and one or more of FLO1, FLO5 and FLO9. The flocculating cells also exhibited a significantly higher hydrophobicity than the non-flocculating strain CBS 8066, which might contribute to the flocculation. The flocculating strain was more tolerant to carboxylic acids and furan aldehydes, but more

sensitive to phenolic compounds. This sensitivity likely depends on the higher cell wall hydrophobicity. Despite the sensitivity to phenolic compounds, the flocculating yeast could easily ferment all hexoses in complete hydrolysate, while the non-flocculating strain was strongly inhibited. The small effect of the inhibitors on the flocculating strain was reflected in small changes in the macromolecular composition of cells grown in different media. Surprisingly, the expression of *YAP1*, *ATR1* and *FLR1*, known to confer resistance against lignocellulose-derived inhibitors, was actually lower in CCUG 53310 than in CBS 8066 in inhibitory media, indicating that the flocculating strain experienced the cultivation conditions as less stressful. We can conclude that *S. cerevisiae* CCUG 53310 is a robust strain suitable for bioethanol production from lignocellulose hydrolysates. Flocculation is likely to confer resistance towards most inhibitors by a high local cell concentration, leading to subinhibitory local levels of inhibitors, causing the cells to experience a lower stress level. This work has a submitted manuscript (Westman et al 2011).

Process simulation

In this work, the process for fuel ethanol production from softwood. The first process developed was based on dilute acid hydrolysis and flocculated yeast fermentation. Then a process based on enzymatic hydrolysis and fermentation using encapsulated yeasts was studied (Fig. 4). For the latter process, wood is pretreated by a cellulose solvent, NMMO, and ethanol is produced by NSSF process. Biogas and solid residues (lignin) are the other valuable by products. Simulation of the process with Aspen Plus was used to solve the process mass and energy balance; finding the bottlenecks of the process, optimizing the equipment configuration, and providing the necessary data for the equipment design. Using mechanical vapor recompression for evaporators in the pretreatment and multi-pressure distillation columns, the energy requirements of the process were minimized. The energy efficiency of the overall process found at the optimum conditions was 79%. The economical feasibility of the developed biorefinery for five different plant capacities was studied by Aspen Icarus Process Evaluator. The base case was designed to utilize 200,000 tons of spruce wood per year and needed M€ 58.3 as total capital investment. Ethanol production yield, based on experiments, was as high as one liter of ethanol per 4 kilograms of wood. Considering biogas and solid residues, the production cost of ethanol is calculated to be 0.44 €/l, while increasing the capacity to four times of the base case reduced it to 0.38 €/l. Effect of other parameters such as, price of feedstock, enzyme, methane, carbon dioxide and solid residue as well as nutrient load on the production cost were investigated as well. After the enzyme price which had been a bottleneck for enzymatic processes, the price of feedstock and solid residues and nutrient load had the most significant impact on the production cost (Shafiei et al. 2011).

Working process of the project

The project has functioned well. We had three PhD students (Johan Westman, Päivi Ylitervo, Hamidreza Barghi and Marzieh Shafiei) and some MSc theses on this subject, while several seniors (Mohammad Taherzadeh, Carl-Johan Franzén and Keikhosro Karimi) were partly involved.

Comments on project goal fulfilment.

We believe we have fulfilled the goals of this project

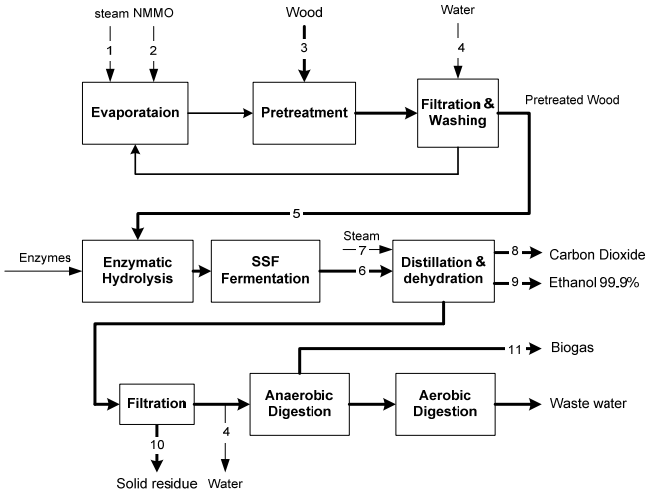

Fig 4. Block flow diagram of the simulated process

Project economy/financing.

The project was in good balance with the budget, which was totally 3,146,000 SEK. Here are the details of the costs:

Costs	2009 (x1000 SEK)	2010 (x1000 SEK)
Personal costs	514	1043
Chemicals, equipments, conferences	91	345
Overhead	212	479
Transferred to Chalmers (Salary Carl-Johan Franzen & materials/equipment)	280	182
Total	**1097**	**2049**

Articles

The project resulted in several publications (and manuscript) in journals and contributions to the relevant scientific conference. Here is the list of three journal publications:

1. Westman J.O., Taherzadeh M.J., Franzén, C.J. (2011): *Flocculation as an important trait for inhibitor tolerance in bioethanol producing Saccharomyces cerevisiae strains*, Submitted
2. Ylitervo, P., Franzén, C.J., Taherzadeh M.J. (2011): *Ethanol production at elevated temperatures using encapsulated yeast*, Manuscript in preparation
3. Shafiei, M., Karimi K., Taherzadeh M.J. (2011): *Techno-economy study of ethanol from spruce by N-methylmorpholine-N-oxide (NMMO) pretreatment*, Manuscript in preparation

Additional comments (if any)

No additional comment