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Ethanol Production from Starch Hydrolyzates using *Zymomonas mobilis* and Glucoamylase Entrapped in Polyvinylalcohol Hydrogel

Martin Rebroš · Michal Rosenberg · Zuzana Grosová · L'udmila Krištofíková · Miroslav Paluch · Matej Šipöcz

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Abstract The glucoamylase from *Aspergillus niger*, immobilized into poly(vinylalcohol) hydrogel lens-shaped capsules LentiKats®, was used for simultaneous saccharification and fermentation (SSF) with *Zymomonas mobilis* in free form. This system was stable in both the repeated batch and continuous mode of SSF. The microorganism was found to adsorb on the capsules with immobilized enzyme. This increased the ethanol productivity of the repeated batch system with 5% *w*/*v* of immobilized glucoamylase almost 2.1 times (7.2 g l^{-1} h^{-1}) compared to free enzyme–free microorganism system (3.5 g l^{-1} h^{-1}). The continuous SSF with the immobilized glucoamylase (11.5% *w*/*v*) tested for 15 days had productivity 10 g l^{-1} h^{-1}, which is comparable to continuous experiments on semi-defined glucose medium (10 g l^{-1} h^{-1}). These two systems were stable in both glucoamylase activity and microorganism productivity.

Keywords Ethanol · Glucoamylase · Immobilization · LentiKats · Poly(vinylalcohol) · *Zymomonas mobilis*

Introduction

Starch, a renewable carbohydrate source of energy, is considered to be a major substrate for ethanol production [1]. Simultaneous saccharification and fermentation (SSF) involves the hydrolysis of polymer into glucose and the conversion to ethanol in the same vessel [2]. The primary advantage of this method, compared to separate hydrolysis and fermentation, is the cost savings resulting from the reduction of the number of reactor vessels needed, the increased rate of hydrolysis due to decreased product inhibition, the reduction of fermentation time, and decreased capital cost [2–5].

An alternative microorganism that might be used in place of yeast in ethanol production processes is *Zymomonas mobilis*. The disadvantage of this bacterium is the lack of

M. Rebroš (✉) · M. Rosenberg · Z. Grosová · L. Krištofíková · M. Paluch · M. Šipöcz
Department of Biotechnology, Faculty of Chemical and Food Technology,
Slovak University of Technology, Radlinského 9, Bratislava 812 37, Slovakia
e-mail: martin.rebros@stuba.sk

glucoamylase activity. Therefore, saccharifying enzyme amyloglucosidase (AMG) should be used together with cells in the SSF processes [6]. In practice, it would be more beneficial if cells and enzyme are capable of being retained at high concentrations in bioreactors or employed repeatedly. The immobilization of these two biocatalysts is one of the most favorable techniques to achieve this goal [7].

One of the recently developed immobilization materials is poly(vinylalcohol) (PVA) hydrogel. This material is the matrix of lens-shaped capsules, LentiKats®, developed by Ding and Vorlop [8]. These capsules were successfully verified with both entrapped *Z. mobilis* biocatalysis [9] and entrapped glucoamylase hydrolysis [10]. The main benefit of glucoamylase immobilized in LentiKats® is the excellent long-term stability of activity in both repeated batch and continuous maltodextrin hydrolysis. This finding promoted our interest in SSF process with glucoamylase immobilized in this form of PVA hydrogel.

In the present study, the saccharification of maltose syrup with entrapped glucoamylase and simultaneous fermentation with *Z. mobilis* in free form was investigated.

Materials and Methods

Z. mobilis CCM 2770 (obtained from Czech Collection of Microorganisms) was stored on the production medium solidified with 2% (*w*/*v*) agar and 100 g l^{-1} of glucose.

Aspergillus niger glucoamylase SAN Extra L (Novozymes, www.novozymes.com) was used in a liquid form (300 U ml^{-1}). One activity unit was defined as the amount of enzyme able to hydrolyze 1 μmol min^{-1} of maltose at 25 °C in 0.1 M acetate buffer (pH 4.3; Novozymes). The SSF experiments were performed on maltose syrup, Glucomalt 551 (consisted of 33% *w*/*v* higher sugars, 20% *w*/*v* maltotriose, 53% *w*/*v* maltose, 4% *w*/*v* glucose; Amylum, Slovakia). The maltose syrup Glucomalt 551 (10% *w*/*v*) is equivalent to 80 g l^{-1} of glucose after complete hydrolysis. The production medium consisted of maltose syrup (12–20% *w*/*v*), 5 g l^{-1} yeast extract, 1 g l^{-1} $(NH_4)_2SO_4$, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1 g l^{-1} KH_2PO_4. The pH was adjusted to 4.5.

Glucoamylase Immobilization

Immobilized glucoamylase was prepared on the pilot-scale equipment in company LentiKat's (Czech Republic, www.lentikats.eu) according to the publication [10] and manufacturer. The enzyme (50 ml) was mixed with 1 l of PVA gel in liquid form, extruded through the thin nozzles on the hard surface and dried down in laminar airflow cabinet to 30% of the initial mass. Solid gel particles (LentiKats®) were swollen in the stabilizing solution according to the manufacturer. Immobilized glucoamylase was stored at 4 °C in acetate buffer (pH 4.5, 10 mM) containing 30% (*w*/*v*) glucose [10].

Batch SSF and SHF with Free Enzyme and Free Microorganism

The inoculum of *Z. mobilis* cells for both experiments were grown in 125 ml production medium with glucose 100 g l^{-1} (250-ml Erlenmeyer flasks) without shaking at 30 °C, until the biomass reached 2 g l^{-1}. Both processes were realized in 3-l fermenter containing 2.5 l of production medium with 12 *w*/*v* of maltose syrup.

The SSF process was inoculated with both microorganism (5% *v*/*v*) and 0.05% *v*/*v* of glucoamylase. Fermentation was carried out at 30 °C, pH 5.0 (automatic addition of 2 M NaOH) with gentle stirring (200 rpm).

The separate hydrolysis and fermentation (SHF) consisted of two separate parts: hydrolysis, to 2.5 l of production medium, the free glucoamylase was added (0.04% *v*/*v*). Hydrolysis was performed at 65 °C for 2 h. Thereafter, the medium was cooled down to 30 °C, production medium was inoculated with 5% *v*/*v* of inoculum, and process was carried out as described above.

Repeated Batch SSF with Immobilized Enzyme and Free Microorganism

Fermenter (1.3 l) containing 1 l of production medium with maltose syrup (20% *w*/*v*) was inoculated with both 25 or 50 g of LentiKats® with immobilized glucoamylase and 100 ml of *Z. mobilis* inoculum (described above). Fermentations were carried out at 30 °C, pH 4.5 (automatic addition of 2 M NaOH) with gently stirring (200 rpm). Each batch cycle was stopped at the time point when the traces of maltotriose and maltose (for the experiment with 50 g of capsules, when the traces of glucose) were in the broth. After each batch cycle, 90% (*v*/*v*) of the medium was separated, and the rest (10% *v*/*v*) was an inoculum for next batch run. This procedure was employed in 21 and 22 repeated batch fermentations, respectively.

Continuous SSF with Immobilized Enzyme and Free Microorganism

Fermenter (1 l) containing 0.5 l of production medium with maltose syrup 13.5 (or 20)% *w*/*v*, was inoculated with both 12.5 g (25.5 or 57.5 g, respectively) of LentiKats® with immobilized glucoamylase and 100 ml of *Z. mobilis* inoculum (described above). Fermentation was carried out at 30 °C, pH 4.5 (automatic addition of 2 M NaOH) and gently stirring (200 rpm). Continuous fermentations had begun at the time point when the residual concentration of glucose decreased to 3 g l^{-1}. All fermentation experiments were duplicated.

Analytical Assays

Free cell biomass in the medium was calculated from the correlation curve between the OD at 620 nm and dry cells weight. Glucose and ethanol were analyzed by high-performance liquid chromatography on the IONEX column in H^+ form (Watrex, Czech Republic); with 9 mM H_2SO_4 as mobile phase, 50 °C and flow rate 0.7 ml min^{-1}.

Results and Discussion

Comparison of SSF and SHF with Free Enzyme and Free Microorganism

Since the optimal temperature of the starch hydrolysis with glucoamylase is 60–65 °C (www.novozymes.com) while the fermentation by *Z. mobilis* is only 30–35 °C, the lower temperature determinates the maximum temperature for SSF process. A typical SSF batch conversion with both free enzyme (0.05% *v*/*v*) and free microorganism (5% *v*/*v*) at 30 °C on maltose syrup (12% *w*/*v*) was completed in 13 h, and the final ethanol concentration 48.7 g l^{-1} (99% of theoretical amount) and ethanol productivity 3.5 g l^{-1} h^{-1} (Fig. 1) was achieved. The glucose maximum (32 g l^{-1}) was reached at the 6 h of the SSF process. Glucose was completely utilized within 10.5 h, but the small amount of maltose and higher sugars were still in the broth. Therefore, the ethanol production was slightly limited by the lack of glucose after this point.

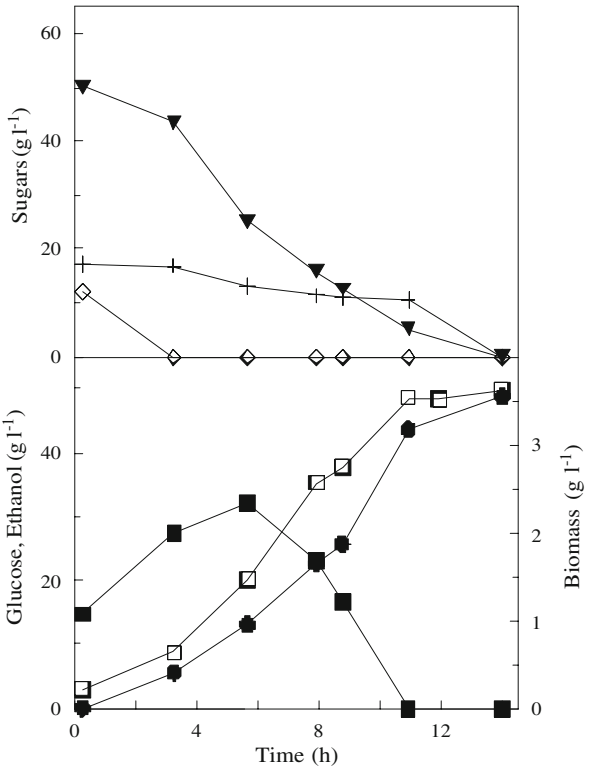

Fig. 1 Batch SSF process with free glucoamylase (0.05% *v/v*) and free *Zymomonas mobilis* on maltose syrup (12% *w/v*). Ethanol (*filled circle*), glucose (*filled square*), maltose (*filled triangle*), maltotriose (*empty diamond*), higher sugars (*plus symbol*), biomass (*empty square*)

Compared to this, the fermentation with pre-hydrolyzed maltose syrup (2 h, 65 °C, and glucoamylase 0.04% *v/v*) took only 11 h, and lower product yields (97%) but higher productivity (4.3 g l^{-1} h^{-1}; Fig. 2) were obtained. The advantage in productivity of SHF is compensated by a higher energy requirement of pre-hydrolyze process and overall duration of the whole process [hydrolysis (2 h), cooling (1.5 h), and fermentation (11 h)]. Furthermore, the maximum specific growth rate (μ_{max}) of *Z. mobilis* in SSF was higher (0.15 h^{-1}) compared to SHF (0.12 h^{-1}), which confirms higher microorganism growth at lower substrate concentration (glucose maximum 32 g l^{-1}).

SSF with Immobilized Enzyme and Free Microorganism in Repeated Batch Mode

The glucoamylase was immobilized into PVA hydrogel particles, LentiKats®, as described before [10]. After immobilization process, the enzyme activity decrease to 35% of its free form activity. Therefore, the amount of immobilized glucoamylase used in immobilized form for the first experiment was 2.9 times higher compared to SSF. Repeated batch cycles were performed for two different amount of immobilized enzyme on maltose syrup (20% *w/v*). The first fermentation of the experiment with 2.3% (*w/v*) of immobilized glucoamylase was inoculated with 10% *v/v* of microorganism in exponential growth phase (Fig. 3). After each batch cycle, 90% (*v/v*) of the medium was separated, and the remaining 10% (*v/v*) was used as inoculum for next batch run. During the first batch run, which took 12 h, the ethanol production rate by the bacteria was slower than the maltodextrin hydrolysis, which resulted in the increase of glucose up to 68 g l^{-1} at 6.6 h of the SSF process (Fig. 3). Each cycle was

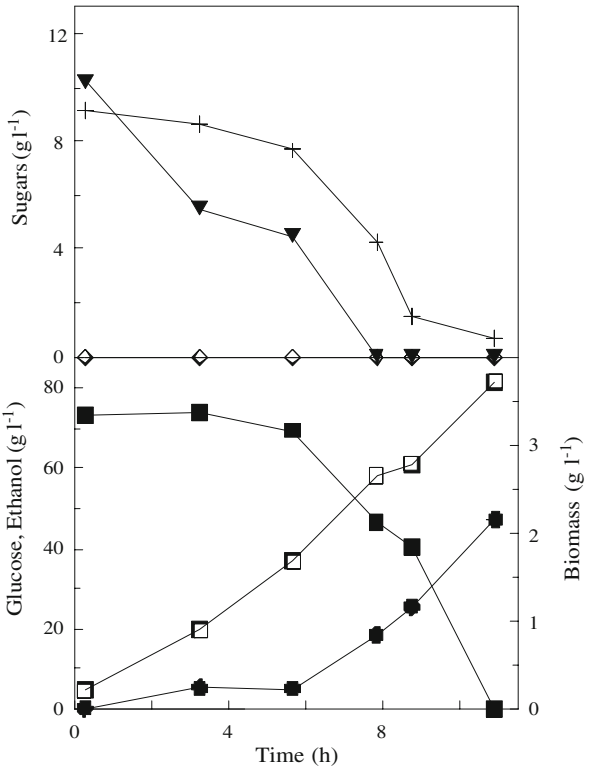

Fig. 2 Batch fermentation with pre-hydrolyzed maltose syrup (2 h, 65 °C, glucoamylase 0.04% *v*/*v*) and free *Zymomonas mobilis* on maltose syrup (12% *w*/*v*). Ethanol (*filled circle*), glucose (*filled square*), maltose (*filled triangle*), maltotriose (*empty diamond*), higher sugars (*plus symbol*), biomass (*empty square*)

completed at the time point when maltotriose and maltose reached trace concentrations in the medium. The glucose maximum in repeated batch fermentations continuously decreased from 68 g l^{-1} (the first fermentation) to 10 g l^{-1} (the 21st fermentation). This indicated increasing activity of the microorganism, which resulted in limitation of fermentation by insufficient glucose production at the end of the process. Moreover, the decrease of productivity from 5.5 g l^{-1} h^{-1} (the first fermentation) to 5 g l^{-1} h^{-1} (the 21st fermentation) was observed.

To avoid glucose limitation of fermentation process, the amount of immobilized enzyme was increased to 5% (*w*/*v*) (Fig. 4). Compared to the previous experiment, the glucose maximum in the first batch conversion increased to 115 g l^{-1}. The glucose peak of each batch run also continuously decreased (the ninth batch, 40 g l^{-1}; the 15th batch 24 g l^{-1}; Fig. 4). Contrasting the previous experiment, each fermentation was stopped when the traces of glucose were in the broth, which eliminated the ethanol production decrease by the lack of the glucose. The amount of immobilized glucoamylase was so high that maltose and maltotriose were completely hydrolyzed at the end of the fermentation. The volumetric ethanol productivity of the batch fermentations increased from 4.9 g l^{-1} h^{-1} (the first fermentation) through 6.8 g l^{-1} h^{-1} (the ninth batch) into 7.2 g l^{-1} h^{-1} (the 15th batch), and remained stable for the next seven batch runs. The average ethanol yield was 84% of the theoretical value. Besides the decrease in biomass concentration in medium (Fig. 4), the microorganism was found to adsorb onto particles with immobilized enzyme. Compared to the weight of capsules at the beginning of experiments, the adsorbed biomass increased the weight of LentiKats® by about 10% (*w*/*v*) after the 22nd batch run.

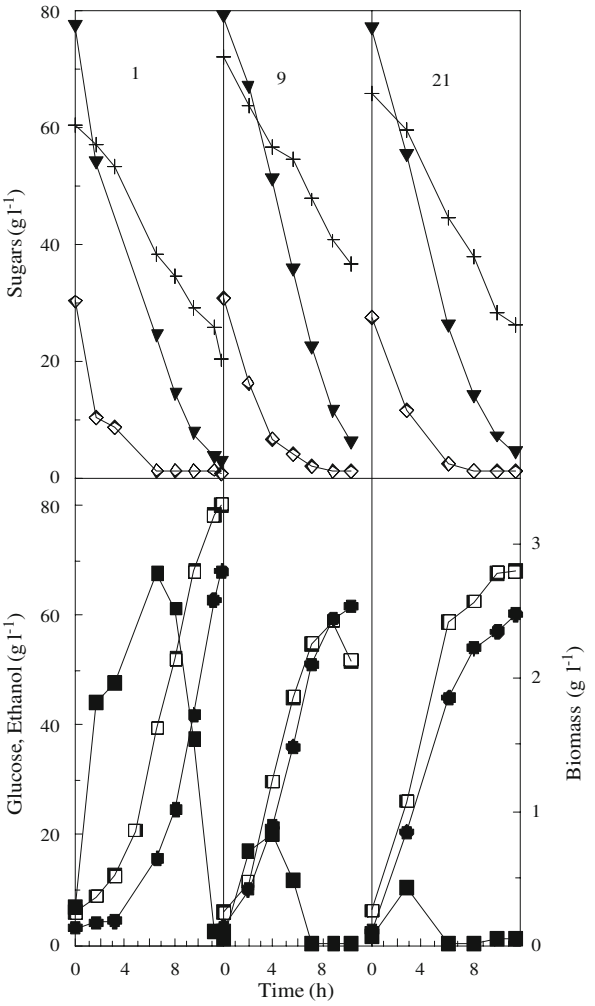

Fig. 3 Repeated batch conversions with glucoamylase immobilized in LentiKats® (2.3% *w/v*) and free *Zymomonas mobilis* on maltose syrup (20% *w/v*; *1* first, *9* 9th, *21* 21st batch conversion). Ethanol (*filled circle*), glucose (*filled square*), maltose (*filled triangle*), maltotriose (*empty diamond*), higher sugars (*plus symbol*), biomass (*empty square*)

The adsorption of microorganisms, first time observed in this work, was also confirmed by microscope examination. The combination of entrapped glucoamylase and both free and adsorbed microorganisms enhanced productivity of SSF almost 1.5 times. Compared to the free enzyme–free microorganism SSF, it was almost 2.1 times (Fig. 1). Kim et al. developed the semi-scale SSF experiments in semi-batch mode (and as described, comparable with lab-scale) in stirred tank reactor (72 l) with free glucoamylase and *Z. mobilis* cells recycle [11]. This fermentation system on 20% sago starch improved the ethanol productivity from 3.17 g l^{-1} h^{-1} (the first batch) to 6.68 g l^{-1} h^{-1} (the fifth batch) with final ethanol 80 g l^{-1} and ethanol yield 88% of theoretical yield [11].

Presented productivities of free microorganism and encapsulated enzyme are even higher compared to submerged fermentations with co-immobilized AMG and *Z. mobilis* in chitin–sodium alginate beads (3.2 g l^{-1} h^{-1}) [12].

The immobilization of enzyme reduces the cost of substrate preparation, and the unnecessary cell recycle reduces the investments in separation (ultrafiltration unit).

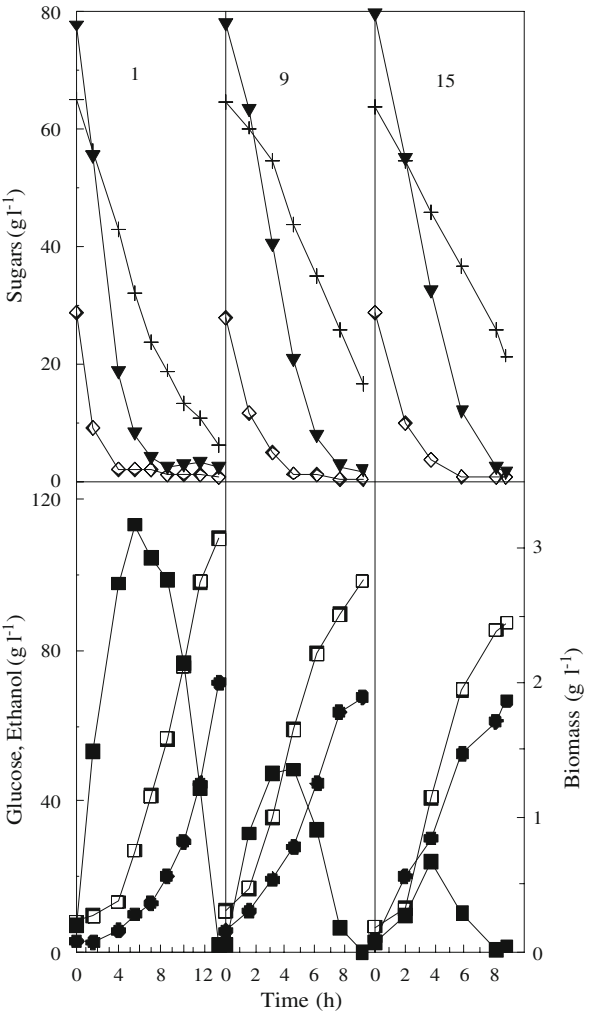

Fig. 4 Repeated batch conversions with glucoamylase immobilized in LentiKats® (5% *w*/*v*) and free *Zymomonas mobilis* on maltose syrup (20% *w*/*v*; *1* first, *9* ninth, *15* 15th batch conversion). Ethanol (*filled circle*), glucose (*filled square*), maltose (*filled triangle*), maltotriose (*empty diamond*), higher sugars (*plus symbol*), biomass (*empty square*)

SSF with Immobilized Enzyme and Free Microorganism in Continuous Mode

As described above, the amount of immobilized enzyme in the SSF process is a limiting factor for effective ethanol production. Because of this, the continuous SSF were investigated at three different concentrations of immobilized glucoamylase [2.5, 5.1, 11.5% (*w*/*v*)]. One approach for continuous SSF, described by several authors, is processing at high dilution rates. This results in high ethanol productivity with low ethanol concentration and low conversion [1, 5, 6]. Avoiding these ineffective process conditions, the conversion of sugars into ethanol was the main parameter for dilution rate setup. The conversion in the experiment with 2.5% (*w*/*v*) of immobilized glucoamylase on maltose syrup [13.5% (*w*/*v*)] at dilution rate 0.27 h^{-1} was 56% (Table 1). The average steady-state ethanol concentration in this process was 27.5 g l^{-1} with no residual glucose and the ethanol productivity of about 7.2 g l^{-1} h^{-1}. The saccharification was a rate-limiting step because insufficient glucose was produced for ethanol conversion.

Table 1 Parameters of continuous SSF with glucoamylase immobilized into LentiKats® and free *Zymomonas mobilis*.

Glucoamylase (% *w/v*)	Feed medium (*w/v*)	D (h^{-1})	Glucose (g l^{-1})	Ethanol (g l^{-1})	Biomass (g l^{-1})	Conversion (%)	Productivity (g l^{-1} h^{-1})	Yield (%)	Tested period (days)
2.5	13.5	0.27	0	27.5	2.5	56	7.2	84	2
5.1	20	0.19	0–3	47	2.6	62	9.1	92	10
11.5	20	0.2	16–22	50.4	2.4	68	10	90	15

To enhance both steady-state ethanol and glucose concentration, the substrate (maltose syrup) was raised to 20% (*w/v*), and immobilized glucoamylase was enhanced to 5.1% (*w/v*). The steady state was reached at the dilution rate of 0.19 h^{-1}, conversion of 62%, and ethanol concentration of about 47 g l^{-1}. The ethanol yield was 92% of the theoretical amount. The steady-state glucose concentration stabilized between 0 and 3 g l^{-1} and ethanol production increased to 9.1 g l^{-1} h^{-1}. The continuous system was tested for 10 days. No drop of ethanol productivity and glucoamylase activity were observed during the experiment.

To increase the conversion and the residual concentration of glucose, the amount of immobilized glucoamylase was increased to 11.5% (*w/v*) in the next experiment. Steady-state dilution rate stabilized at 0.2 h^{-1} at conversion of 68% and ethanol concentration of about 50.4 g l^{-1}, and glucose varied between 16 and 22 g l^{-1}. The ethanol productivity increased to 10 g l^{-1} h^{-1}. The ethanol yield slightly decreased to 90% of the theoretical amount. This system, tested for 15 days, was stable without any changes in both ethanol productivity and glucoamylase activity.

The results of continuous SSF process correspond to free *Z. mobilis* continuous fermentation on glucose medium (150 g l^{-1}), which had almost the same ethanol productivity (10.5 g l^{-1} h^{-1}) [9]. Besides the observed adsorption of microorganism onto capsules with immobilized glucoamylase in continuous mode of SSF (confirmed by microscope), the expected increase in ethanol productivity was not noticed. We assume that the high ethanol concentration inhibited the ethanol production of *Z. mobilis* in steady state [13]. The presented results of continuous SSF are also comparable to the SSF process in fluidized-bed reactor with co-immobilized glucoamylase and *Z. mobilis* in κ-carrageenan (66.7% *v/v*) [1]. These authors reported that this type of reactor had productivity of 12.2 g h^{-1} l^{-1} on synthetic maltodextrin feed (188 g l^{-1}) at dilution rate of 0.27 h^{-1}. At these conditions, conversion of 53.1% was reached with ethanol 45.3 g l^{-1}. Compared to this, the higher conversion (68%) with higher ethanol concentration (50.4 g l^{-1}) and comparable productivity (10 g l^{-1} h^{-1}) were achieved in stirred reactor filled with 11.5% *w/v* of immobilized glucoamylase and free microorganism. This confirms that the stirred reactor configuration is effective for SSF process. Moreover, the application of this system in industry is more convenient because stirring reactor conformations are traditionally used in distilleries. In addition, they are less expensive compared to fluidized-bed reactors. Furthermore, budget saving is linked also to the cost of immobilized glucoamylase preparation (11.5 *w/v* compared to 66.7% *v/v*).

It is obvious that the amount of immobilized glucoamylase is crucial in both the repeated batch and continuous SSF process. Compared to the repeated batch experiments, the increase of immobilized glucoamylase up to 5% (*w/v*) in continuous fermentation was insufficient to produce enough glucose for the fermentation process. The advantage of

higher ethanol productivity in continuous system is counterbalanced by the lower conversion and higher residual concentration of maltose, maltotriose, and higher sugars compared to the repeated batch fermentations. From this point of view, the repeated batch conversions seem to be more effective. However, the simple operation of the continuous system is the advantage in long-term testing of operational stability of SSF system in laboratory scale.

Conclusions

The immobilization of glucoamylase in the polyvinylalcohol hydrogel capsules LentiKats® holds this enzyme within the reactor, which can be applied for SSF process with *Z. mobilis*. The immobilized glucoamylase was found to be stable in both the repeated batch and continuous operation of SSF. The microorganism did not interfere with the activity of enzyme. The system in repeated fermentation mode was stable during 21 (immobilized glucoamylase, 2.5% *w*/*v*) and 22 (immobilized glucoamylase, 5% *w*/*v*) repeated batches without changes in ethanol productivity and glucoamylase activity.

Microorganism was found to adsorb onto the particles, which increased the productivity in this fermentation mode almost 1.5 times. The continuous system of SSF had higher ethanol productivity (9 g l^{-1} h^{-1}) for 5.1% *w*/*v* of immobilized glucoamylase. The main disadvantage of the continuous system, compared to the repeated batches, is the lower conversion of substrate into glucose (61% for 5.1% *w*/*v* of immobilized glucoamylase). The increase in conversion (68%) was reached by the increasing immobilized glucoamylase to 11.5% *w*/*v*. This enhancement in glucoamylase resulted in the increase of productivity (10 g l^{-1} h^{-1}), which is comparable to continuous experiments on semi-defined glucose medium [9].

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References

1. Krishnan, M. S., Nghiem, N. P., & Davison, B. H. (1999). *Applied Biochemistry and Biotechnology*, *77*, 359–372. doi:10.1385/ABAB:78:1-3:359.
2. Spangler, D. J., & Emert, G. H. (1986). *Biotechnology and Bioengineering*, *28*, 115–118. doi:10.1002/bit.260280118.
3. Hinman, N. D., Schell, D. J., Riley, C. J., Bergeron, P. W., & Walter, P. J. (1992). *Applied Biochemistry and Biotechnology*, *34*, 639–649. doi:10.1007/BF02920584.
4. Eklund, R., & Zacchi, G. (1995). *Enzyme and Microbial Technology*, *17*, 255–259. doi:10.1016/0141-0229(94)00014-I.
5. Lee, G. M., Kim, C. H., Abidin, Z., Han, M. H., & Rhee, S. K. (1987). *Journal of Fermentation Technology*, *65*, 531–535. doi:10.1016/0385-6380(87)90112-9.
6. Kim, C. H., Lee, G. M., Abidin, Z., Han, M. H., & Rhee, S. K. (1988). *Enzyme and Microbial Technology*, *10*, 426–430. doi:10.1016/0141-0229(88)90037-3.
7. Fukui, S., & Tanaka, A. (1982). *Annual Review of Microbiology*, *36*, 145–172. doi:10.1146/annurev.mi.36.100182.001045.
8. Ding, W. A., & Vorlop, K. D. (1995). Patent DE 4327923.
9. Rebroš, M., Rosenberg, M., Stloukal, R., & Krištofíková, L'. (2005). *Letters in Applied Microbiology*, *41*, 412–416. doi:10.1111/j.1472-765X.2005.01770.x.
10. Rebroš, M., Rosenberg, M., Mlichová, Z., Krištofíková, L'., & Palúch, M. (2006). *Enzyme and Microbial Technology*, *39*, 800–804. doi:10.1016/j.enzmictec.2006.01.001.

11. Kim, C. H., Abidin, Z., Ngee, C. C., & Rhee, S. K. (1992). *Bioresource Technology*, *4*, 1–6. doi:10.1016/0960-8524(92)90111-A.
12. Bandaru, V. V. R., Somalanka, S. R., Mendu, D. R., Madicherla, N. R., & Chityala, A. (2006). *Enzyme and Microbial Technology*, *38*, 209–214. doi:10.1016/j.enzmictec.2005.06.002.
13. Sun, M. Y., Nghiem, N. P., Davison, B. H., Webb, O. F., & Bienkowski, P. R. (1998). *Applied Biochemistry and Biotechnology*, *70*, 429–439. doi:10.1007/BF02920158.