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One-step hydrolysis ethanol fermentation of cellobiose and pinewood-cellulose by cell factories of non-GMO *Saccharomyces cerevisiae* using kissiris and γ-alumina as support

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Abstract

This project aimed to compare the efficiencies of cell factories designed using non-engineered cells of *Saccharomyces cerevisiae* to conduct a one-step process of simultaneous hydrolysis and fermentation of cellobiose to ethanol. The investigation involved the preparation of CFs of *S. cerevisiae* cells covered by starch gel and mineral kissiris or γ-alumina with a cellulolytic agent (*Trichoderma reesei*, or a commercial cellulase, or our lab-preparation of cellulase). Analysis showed that high ethanol yield (up to 82%) was achieved in a 72-hours one-step process using CFs *S. cerevisiae*/SG-commercial cellulases, and *S. cerevisiae*-alu kis/SG-commercial cellulases, indicating the efficiency of CFs. Thereafter, the CF with the best performance was used for cellulose to bioethanol in one-step resulting in 61.7% cellulose bioconversion. In our studies, the ethanol yield and substrate bioconversion were found competitive, with another research reported using engineered *S. cerevisiae* and co-immobilization. We used non-engineered yeast cells, cheaper material kissiris, and our lab-preparation of cellulases.

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Introduction

In the last few decades, excessive exploitation of fossil fuels has occurred, as a result of the growing population and rising living standards [1,2]. This over-exploitation has increased environmental problems - such as global warming, climate change, and scarcity of fossil fuels [3]. Therefore, finding economical, environment-friendly, and renewable alternative sources for energy generation is essential [4,5]. From this perspective, using lignocellulosic biomass as a raw material for second-generation bioethanol production is an alternative strategy [6,7]. Bioconversion of a variety of cellulosic biomass to ethanol requires two bioprocesses including enzymatic hydrolysis, and fermentation of produced sugars [8]. However, *S. cerevisiae* can ferment sugars, but not cellulose and cellobiose [9,10]. This drawback is due to the lack of the β-glycosidase enzyme [11]. Recently, scientists have been researching ways to overcome this problem. Recombinant *S. cerevisiae* strains that express cellulases were used to enable the fermentation of cellulose and cellobiose [12,13]. An alternative strategy was the co-culture of *S. cerevisiae* with bacterium or fungus [14]. Recently researchers studied the use of enzyme mimicking features of nanoparticles coupled with yeast for ethanol fermentation [15]. However, one-step simultaneous hydrolysis and fermentation (OSHF) without the use of recombinant *S. cerevisiae* is more convenient for industrial bioethanol production as genetically modified organism (GMO) yeast has not been used for industrial ethanol production [16,17]. Cell factories (CFs) without GMO were used for the OSHF process, *S. cerevisiae/BC-A. awamori* for bioconversion of starch [18], and *S. cerevisiae/SG*-cellulases for cellobiose [11]. The advantage of these CFs can be for the production of other value-added chemicals, with the use of the appropriate microorganism and without any environmental concerns with the use of GMO [19,20].

In present study, starch gel (SG), γ-alumina (alu), and mineral kissiris (kis) were used for immobilization of *S. cerevisiae* as the first layer of CF, which will ferment cellobiose in OSHF. Starch an abundant low-cost natural material was selected for SG preparation, as this biopolymer has been found not affected in the bioprocess [21]. Kis and alu solid inert materials have been found ideal supporting materials for the immobilization and promoters of fermentation [22,23]. Kis has been used as a support material in the industrial scale 100,000 L bioreactor [24]. Kis is inert, abundant cheaper material, and can also be regenerated for reuse [25,26].

Therefore, this investigation aimed to compare the performance of several designs of CF prepared with SG, kis, and alu as carriers of non-engineered *S. cerevisiae* in OSHF, first using cellobiose as a model substrate, and then the best performing CF to be applied for OSHF of cellulose.

Materials and methods

Materials

Chemicals used were - Starch (Penta, Czech Rep.); tri-sodium citrate 2 a.q. (Chem-lab, Belgium); citric acid (Acros Organics, USA); cellobiose (Alfa Aesar by Thermo Fischer Scientific); glucose, ethanol, and methanol (Fisher Scientific, UK); 2-propanol (Merck, Germany); PDA (Conda, Spain).

Microorganisms, medium and enzyme

The commercial baker yeast *S. cerevisiae* Lesaffre Hellas "L'hirondelle" product was used in designing of CFs for OSHF.

T. reesei (DSM No. 768) was supplied by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. It was grown on Potato Dextrose Agar (PDA) medium containing 15 g/L agar, 20 g/L dextrose, and 4 g/L, potato extract at 30°C for 6 days. After sporulation, the spores were aseptically collected from PDA slants and counted using a hemocytometer-Neubauer Improved, HBG, Germany.

Commercial cellulase from *Trichoderma reesei* ≥ 700 units/g was obtained from Sigma.

Preparation of cellulose from pinewood

Sawdust of pinewood was obtained from a local timber industry. The delignification procedure was carried out by slow heating sawdust at 70°C with a mild (1%, w/v) NaOH solution for 3 hours. The ratio of sawdust to NaOH solution was 1:10 (w/v) and the volume of the mixture was kept constant during the heating process by the addition of water. After treatment, the delignified sawdust was washed several times with warm deionized water until solubilized lignin residues and NaOH solution were completely removed. Delignified sawdust was dried, stored at room temperature, and used in experiments as cellulose [21].

Preparation of lab-produced cellulases (lc)

Seed culture: Spores of *T. reesei* grown on PDA slants were collected with sterilized deionized water to prepare a spore suspension of 10^7–10^8 spores/mL. One mL of spore suspension and 30 mL medium containing 20 g/L cellulose, 10 g/L peptone, 10 g/L glucose, and pH 4.5 were transferred into 250 mL Erlenmeyer flasks. Flasks were incubated for 24 h at 28°C with agitation at 180 rpm.

Cellulase production in bioreactor: A 5% (v/v) of seed culture (6 x 10^6 spores/mL) and 1L culture medium (containing 50 g/L cellulose, 17 g/L soya peptone, 5 g/L $(NH_4)_2SO_4$, 6 g/L KH_2PO_4, 1 g/L $MgSO_4$, 2.5 g/L glycerol, and 2 mL/L Tween-20, pH 5.0) were aseptically transferred into 2-L stirred fermenter (Electrolab Biotech Ltd.). The culture conditions were maintained at agitation speed 300 rpm, aeration rate 3 L/min, temperature 26°C. After 5 days of incubation, the culture-filtrate was collected by centrifugation at 5000 rpm for 10 min and used as the lab preparation of cellulase [21].

Starch gel production

2 g starch was added to 25 mL deionized water under stirring and heated at 90°C to obtain SG. The mixture was left to cool down at room temperature.

Immobilization of *S. cerevisiae* on kissiris or alumina

Following materials were placed in a conical flask - 30 g mineral kissiris, or cylindrical γ-alumina pellets (γ-Al_2O_3, AKZO, Alumina Extrudates, HDS-000-1.5 mm E, 250 $m^2 g^{-1}$ specific surface area, 10 mm length, 1.5 mm diameter, 0.7 cm^3/g pore volume) with 100 mL of sterilized culture medium (1 g/L KH_2PO_4, 1 g/L $(NH_4)_2S0_4$, 5 g/L $MgSO_4$, 2 g/L yeast extract and 110 g/L glucose, pH 4.8) and 6.25 g *S. cerevisiae*. The culture was incubated at 30°C and allowed to ferment until the hydrometer density (°Be) was equal to 0.5. The supernatant was removed and the pieces of kis or alu with immobilized yeast cells were collected [22,27].

Cell factories production for cellobiose OSHF

Starch gel cell factories

1) 7.5 mL *T. reesei* spores' suspension (30 x 10^6/mL), or cellulase 100 FPU/g, or 25 mL lab-produced cellulase was added drop by drop to 25 mL of SG under stirring. Then, 6.25 g of compressed baker's yeast were dispersed on a plate and the SG-cellulolytic agent mixture was added drop by drop to cover the layer of *S. cerevisiae*. Three different SG CFs were prepare *S. cerevisiae*/SG-*T. reesei* (Sc/SG-Tr),

2) *S. cerevisiae*/SG-commercial cellulase (Sc/SG-cc),

3) *S. cerevisiae*/SG-lab-produced cellulases (Sc/SG-lc).

Kissiris-starch gel cell factories

7.5 mL *T. reesei* spores' suspension (30 x 10^6/mL), or cellulase 100 FPU/g, or 25 mL lab-produced cellulases was added drop by drop to 25 mL of SG under stirring. Then, the immobilized *S. cerevisiae* on kissiris was dispersed on a plate and the SG-cellulolytic agent mixture was added drop by drop to cover the layer of kissiris. Three different kis/SG CFs were prepared:

1) *S. cerevisiae*-kis/SG-*T. reesei* (Sc-kis/SG-Tr),

2) *S. cerevisiae*-kis/SG-commercial cellulase (Sc-kis/SG-cc),

3) *S. cerevisiae*-kis/SG-lab-produced cellulases (Sc-kis/SG-lc).

Alumina-starch gel cell factories

The process similar to kissiris. was followed using γ-alumina instead of kissiris. Three different alu/SG CFs were prepared:

1) *S. cerevisiae*-alu/SG-*T. reesei* (Sc- alu/SG-Tr),

2) *S. cerevisiae*-alu/SG-commercial cellulase (Sc-alu/SG-cc),

3) *S. cerevisiae*-alu/SG-lab-produced cellulases (Sc-alu/SG-lc).

Cell factory used for cellulose OSHF

Starch in the quantity of 8 g was added in 100 mL deionized water and heated at 90°C for 5 min, then left to cool at room temperature and 100 mL commercial cellulase (100 FPU) was mixed with the prepared gel. Finally, the SG/commercial cellulases biocatalyst was added dropwise on 15 g compressed baker's yeast and the cell factory of *S. cerevisiae*/SG-cellulases was prepared.

Cellobiose OSHF

70 g/L cellobiose were taken each in nine sets of triplicate Erlenmeyer flasks with nine designs of CFs prepared in sections above, 50 mL citrate buffer pH 5, OSHF performed at 30 °C under gentle stirring for three days.

Pinewood-Cellulose OSHF

25 g/L cellulose (delignified pinewood) was taken into Erlenmeyer flask with CF prepared in section above, 200 mL citrate buffer pH 5, OSHF performed at 30°C under gentle stirring for ten days.

Analytical methods

Analysis of cellobiose, glucose, and ethanol was performed by High-Pressure Liquid Chromatography (Shimadzu LC-9A with Nucleogel Ion 300 OA column, LC-9A pump, RID-6A refractive index detector, CTO-10A column oven, and DGU-2A degassing unit). An aqueous solution of 0.017 M H_2SO_4 as the mobile phase (flow 0.55 mL/min), and 1% v/v 2-propanol as internal standard was used. The column temperature was 33°C. The injection volume was 40 µL of 1 % v/v dilution. The cellobiose, glucose, and ethanol concentrations were calculated using standard curves [11].

Calculations of conversion, ethanol yield, and productivity

The following calculations were made as [11]:

$$Ethanol\ yield\ (\%) = \frac{actual\ ethanol\ concentration\ (g_{EtOH}/l)}{theoretical\ ethanol\ concentration\ (g_{EtOH}/l)} x100 \quad (1)$$

$$Ethanol\ productivity\ (g/l/h) = \frac{maximum\ ehtnaol\ concnetration\ (g_{EtOH}/l)}{fermentation\ time\ (h)} \quad (2)$$

$$Conversion\ (\%) = \frac{maximum\ ethanol\ concentration\ (g_{EtOH}/l)}{0.51 \times initial\ cellobiose\ concentration\ (g_{cellob}/l)} \times 100 \quad (3)$$

Results and discussions

Rational

The experimental design of this study was focused on comparing CFs with different materials as immobilization carriers and cellulolytic agents for cellobiose fermentation in OSHF. The best performer CFs, Sc/SG-cc and Sc-kis/SG-cc for cellulose and cellobiose OSHF could be useful to promote further research on improving yield, productivity, and final ethanol concentration on large-scale OSHF of cellulose for second-generation bioethanol production. CFs containing immobilized *S. cerevisiae* on SG, kis or alu and covered by a thin layer of entrapped cellulolytic agents (*T. reesei*, commercial or lab-produced cellulases) in SG were used for cellobiose OSHF. Then, the CF of Sc/SG-cc with higher resulting cellobiose conversion and ethanol yield was used for cellulose OSHF. Previous work [11] has shown that the use of freeze-dried CF led to high cellobiose conversion. In this study, the CFs production did not include the process of freeze-drying reducing the overall production cost, still, CFs produced similar results to the previous report using freeze-dried CFs. The current approach gives the possibility of using non-GMO yeast for cellulose fermentation. This model of CF using other microorganisms and/or enzymes could be used for the production of several other value-added chemicals or biofuels in the frame of white biotechnology [28,29]. Based on a similar principle, specifically designed CFs for OSHF of lactose and starch were used [18,30]. This investigation was done to find the best performer design of CF for cellulose OSHF, using supports kis and alu, which have been used in industrial-scale fermentation [24].

Performance of CFs Sc/SG-cellulolytic agents

Figure 1 illustrates the kinetics of ethanol production and substrate consumption in cellobiose OSHF by 3 designs of CF using either *T. reesei*, or commercial (cc), or lab-produced (lc) cellulases as hydrolysis agents. The cellobiose was completely consumed in CF sets of Sc/SG-cc compared to CFs of Sc/SG-Tr and Sc/SG-lc. Ethanol was produced at 3.6 mL/L and 37 mL/L after 72 h OSHF with CF Sc/SG-Tr and Sc/SG-cc, respectively. Though 8.86 mL/L ethanol was produced within 48 h with CF of Sc/SG-lc. Results presented in Table 1 show the CF Sc/SG-cc gave a higher ethanol yield and cellobiose conversion (82%) than CFs designed with other sources of the enzyme, Sc/SG-Tr and Sc/SG-lc. The maximum ethanol concentration was achieved in 72 h using CF Sc/SG-cc (Figures 1 & 4). The results indicate that the commercial cellulase using SG as an immobilization carrier significantly improved the kinetics of cellobiose fermentation in OSHF at 30°C. While the cellobiose was partially hydrolyzed *by T. reesei* and lab-produced cellulases.

Table 1: Parameters of cellobiose OSHF using CF with non-engineered *S. cerevisiae* and cellulolytic agent (*T. reesei*/cc/lc) immobilized on SG/kis/alu.

	Initial cellobiose (g/L)	Final cellobiose (g/L)	Fermentation time (h)	Ethanol				Final glucose (g/L)	Ethanol productivity (g/L/h)	Ethanol yield (%)	Cellobiose Conversion (%)
				Ethanol from fermentation		Total ethanol					
				mL/L	g/L	mL/L	g/L				
Cell factory with SG											
Sc/SG-Tr	70.00 ± 0.00	46.03 ± 3.79	72	3.64 ± 0.47	2.87 ± 0.37	3.64 ± 0.47	2.87 ± 0.37	5.46 ± 5.46	0.04 ± 0.01	8.05 ± 1.04	8.05 ± 1.04
Sc/SG-cc	70.00 ± 0.00	0.00 ± 0.00	72	37.06 ± 1.32	29.24 ± 1.05	37.06 ± 1.32	29.24 ± 1.05	19.38 ± 17.21	0.41 ± 0.01	81.90 ± 2.93	81.90 ± 2.93
Sc/SG-lc	70.00 ± 0.00	44.99 ± 1.34	48	8.86 ± 1.18	6.99 ± 0.93	8.86 ± 1.18	6.99 ± 0.93	0.00 ± 0.00	0.15 ± 0.02	19.59 ± 2.61	19.59 ± 2.61
Cell factory with kis-SG											
Sc-kis/SG-Tr	70.00 ± 0.00	46.82 ± 0.08	36	6.54 ± 3.87	5.16 ± 3.06	16.37 ± 0.38	12.92 ± 0.30	0.00 ± 0.00	0.27 ± 0.01	36.18 ± 0.84	14.46 ± 8.56
Sc-kis/SG-cc	70.00 ± 0.00	0.00 ± 0.00	48	17.10 ± 3.72	13.49 ± 2.94	26.73 ± 0.71	21.09 ± 0.56	1.08 ± 0.18	0.59 ± 0.02	59.08 ± 1.58	37.79 ± 8.23
Sc-kis/SG-lc	70.00 ± 0.00	37.28 ± 1.63	24	9.52 ± 0.87	7.51 ± 0.69	15.75 ± 2.09	12.43 ± 1.65	6.78 ± 1.98	0.26 ± 0.03	34.81 ± 4.62	21.03 ± 1.92
Cell factory with alu-SG											
Sc-alu/SG-Tr	70.00 ± 0.00	41.43 ± 10.40	48	5.36 ± 3.36	4.23 ± 2.65	13.74 ± 1.24	10.85 ± 0.97	0.00 ± 0.00	0.30 ± 0.03	30.36 ± 2.72	11.85 ± 7.42
Sc-alu/SG-cc	70.00 ± 0.00	12.57 ± 12.57	24	18.07 ± 0.67	14.26 ± 0.53	36.46 ± 4.15	28.763 ± 3.27	2.01 ± 2.01	0.60 ± 0.06	80.57 ± 9.16	39.94 ± 1.49
Sc-alu/SG-lc	70.00 ± 0.00	32.47 ± 7.33	48	8.54 ± 3.75	6.74 ± 2.96	17.17 ± 0.71	13.54 ± 0.56	2.01 ± 2.01	0.56 ± 0.2	37.94 ± 1.56	18.88 ± 8.29

Performance of CFs Sc-kis/SG-cellulolytic agents

Figure 2 illustrates the kinetics of ethanol production and substrate consumption in cellobiose OSHF by 3 designs of CF *Sc*-kis/SG-cellulolytic agents either *T. reesei*, or cc, or lc as hydrolysis agents. The ethanol was produced at a maximum level of 16.4 mL/L after 36 h, 26.7 mL/L after 48 h, and 15.7 mL/L after 24 h with Sc-kis/SG-Tr, Sc-kis/SG-cc, and Sc-kis/SG-lc, respectively. The ethanol yield and cellobiose conversion ranged between 34.8-59% and 14.5-37.8%, respectively (Table 1), though the maximum ethanol yield and cellobiose conversion were achieved with CF Sc-kis/SG-cc. The presence of glucose was detected due to the hydrolysis of cellobiose to glucose by the cellulolytic agents. Cellobiose was completely consumed in OSHF by CF Sc-kis/SG-cc but the ethanol yield was low (26.7 mL/L).

Figure 2 shows the kinetics of cellobiose fermentation with the effect of *S. cerevisiae* immobilization process. Ethanol production is observed during the process due to the conversion of glucose added to the immobilization liquid medium, to ethanol. After immobilization and CF preparation, centrifugation followed to obtain the final CF. However, the centrifugation did not remove all the ethanol produced, thus a small amount of ethanol was transferred with the CF to the cellobiose fermentation medium. This amount of ethanol (6.2-9.8 mL/L) is observed in the figure on day 0. Therefore, the ethanol shown in Figure 2 & 4 consists of the ethanol produced in immobilization process and OSHF.

Performance of CFs Sc-alu/SG-cellulolytic agents

Figure 3 illustrates the kinetics of ethanol production and substrate consumption in cellobiose OSHF by 3 designs of CF Sc-alu/SG-cellulolytic agents either *T. reesei*, or cc, or lc as hydrolysis agents. Ethanol was produced at 13.7 mL/L and 17.2 mL/L after 48 h of OSHF with CF Sc-alu/SG-Tr and Sc-alu/SG-lc, respectively. Though 36.5 mL/L ethanol was produced within 36 h with CF Sc-alu/SG-cc (Figures 3 & 4). Results in Table 1 show the CF Sc-alu/SG-cc reached higher ethanol yield (81%) and cellobiose conversion (40%), compared to CFs Sc-alu/SG-Tr, and Sc-alu/SG-lc (30.4 - 37.4% yield and 11.8 - 18.9% conversion). The cellobiose was partially consumed and the glucose from cellobiose hydrolysis was partially fermented in all cases. The maximum ethanol concentration of 36.5 mL/L was achieved in 36 h using Sc-alu/SG-cc (Figures 3 and 4). The results indicate that the commercial cellulase using alu as an immobilization carrier significantly improved the kinetics of cellobiose OSHF at 30°C. While the cellobiose was partially hydrolyzed by CF containing *T. reesei* and lc cellulases.

Figure 3 shows the kinetics of cellobiose fermentation from day 0 to day 3. Also shows the effect of *S. cerevisiae* immobilization process on ethanol and glucose concentration (immobilization to day 0 in the figure). Ethanol production is observed during the process due to the conversion of glucose, added to the immobilization liquid medium, to ethanol. After immobilization and CF preparation, centrifugation followed to obtain the final CF. However, the centrifugation did not remove all the ethanol produced, thus a small amount of ethanol was transferred with the CF to the cellobiose fermentation medium. This amount of ethanol (8.4-18.3 mL/L) is observed in the figure on day 0. Therefore, the ethanol shown in figures 3 & 4 consists of the ethanol produced by the immobilization process and fermentation.

Cellulose OSHF by non-GMO CF Sc/SG-cc

Figure 5 illustrates the kinetics of bioethanol production in cellulose fermentation by CF *Sc*/SG-cc. Ethanol was produced at 6.3 mL/L after 48 h of OSHF and at 86% after 24 h which gradually decreased after 48 h. This is due to the significant reduction in the hydrolysis rate of cellulose. The cellulose conversion reached 61.2%. The results indicate that the Sc/SG-cc can ferment cellulose to a significant level in 48 h OSHF process.

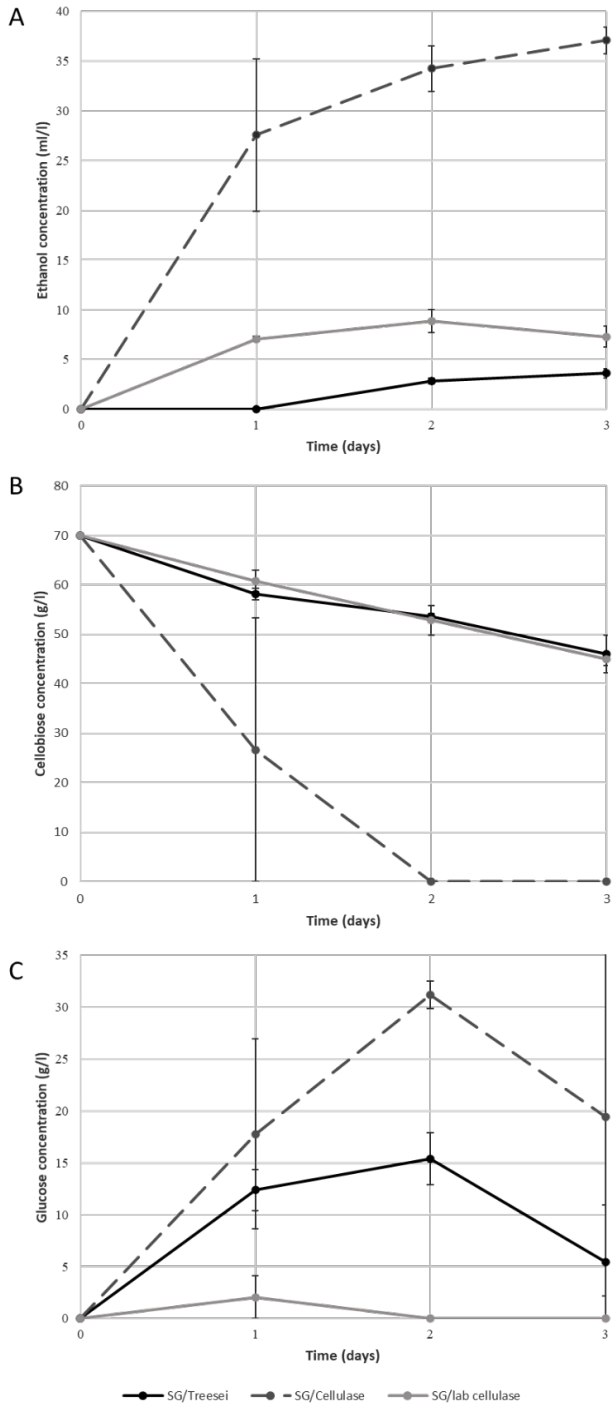

Figure 1: Fermentation kinetics of 7% cellobiose OSHF using CF of Sc/SG-Tr, Sc/SG-cc, Sc/SG-lc .

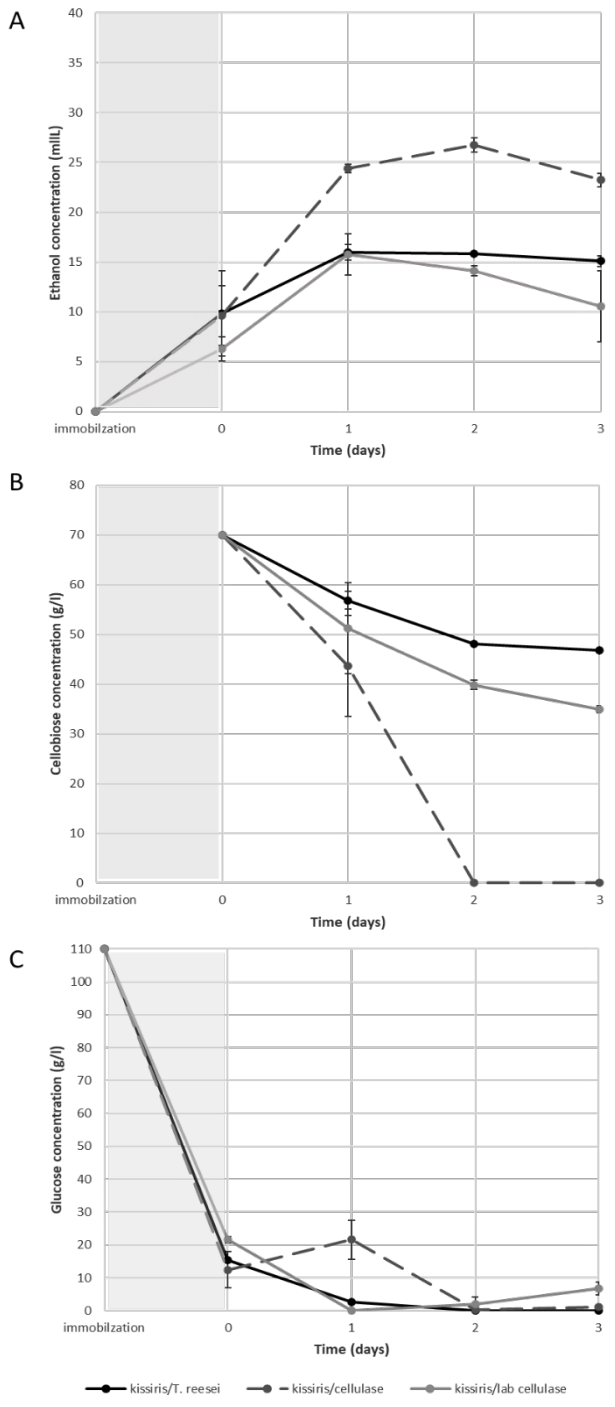

Figure 2: Fermentation kinetics of 7% cellobiose OSHF using CF of Sc-kis/SG-Tr, Sc-kis/SG-cc, Sc-kis/SG-lc.

Scientific and technological consideration of results

A comparison of the results obtained from the use of the CFs of non-GMO *S. cerevisiae* for the OSHF of cellobiose using SG, kis, or alu as immobilization carriers and *T. reesei*, commercial or lab-produced cellulases as cellulolytic agents was conducted. The Sc/SG-cc CF was more efficient to ferment 70 g/L cellobiose at 30°C, producing 37 mL/L ethanol with 82% ethanol yield in 72 h. Moreover, the results indicated that CF Sc-alu/SG-cc ethanol efficiency was similar to CF Sc/SG-cc but with a lower cellobiose conversion. However, ethanol productivity for the CF with alu was 0.90 g/L.h instead of 0.41 g/L.h for Sc/SG-cellulase. CFs showed suitability for bioconversion of cellulose and cellobiose medium and can be used to process glucose and cellobiose mixtures in liquid and solid wastes from food and agro-industries.

SG, alu, and kis were used as support materials for *S. cerevisiae* immobilization. They are inert, cheap, and abundant materials [21,25,26] and promote alcoholic fermentation [31,32]. The results show that SG performed better as an immobilization support material for OSHF than kis and alu, due to all cellobiose being converted. The SG CF produced a higher yield and conversion, but lower productivity than alu CF. Probably, the immobilized cells in the SG were in a steadier environment and there was no risk of cell loss by the turbulence caused by agitation and CO_2 emission. Nevertheless, the use of SG as immobilization support has some economic disadvantages instead to kis and alu cheaper materials than SG. In this study, the selection of the CF for cellulose OSHF was made based on the effectiveness of substrate bioconversion to ethanol.

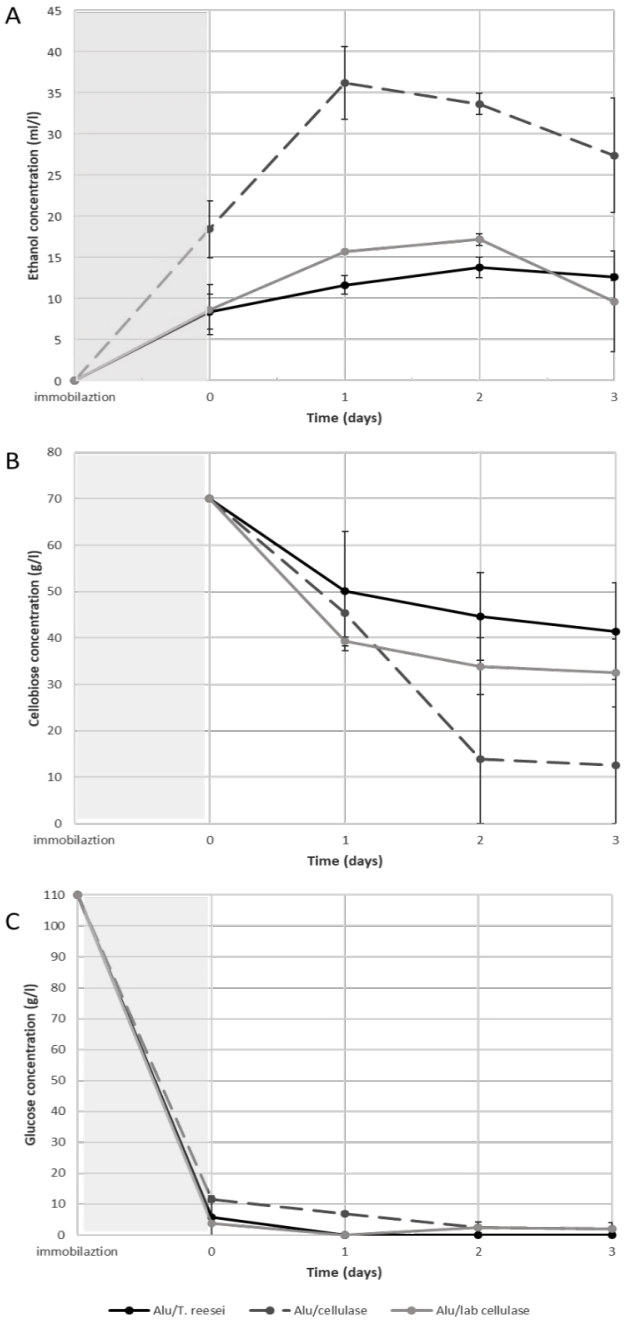

Figure 3: Fermentation kinetics of 7% cellobiose OSHF using CF of Sc-alu/SG-Tr, Sc-alu/SG-cc, Sc-alu/SG-lc.

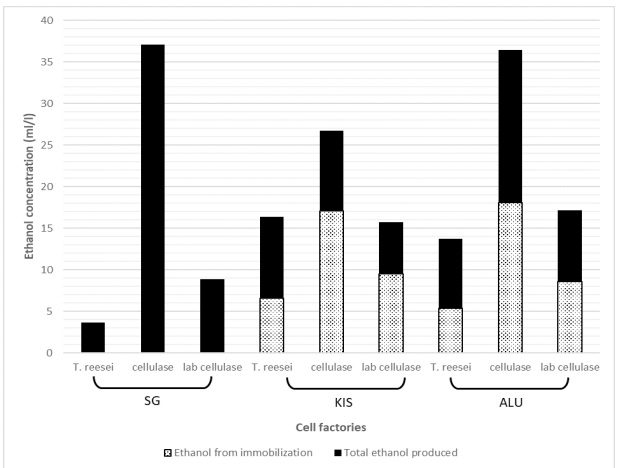

Figure 4: Ethanol produced in OSHF of 7% cellobiose by SG, Kis/SG, and Alu/SG CFs with non-engineered *S. cerevisiae* and *T. reesei*, cc, and lc.

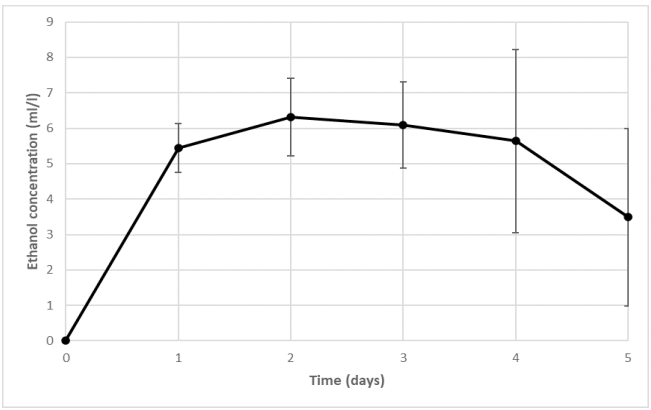

Figure 5: Fermentation kinetics of OSHF of cellulose (2.5%) by CF Sc/SG-cc.

The results showed that commercial cellulases were better cellulolytic agent than *T. reesei* and lab-produced cellulases. The use of cc led to higher ethanol yield and cellobiose conversion in all cases. *T. reesei* could be less efficient as it had to first produce the cellulase. Furthermore, when spores of *T. reesei* are used directly, it is often observed feedback inhibition in the cellulase synthesis and hydrolysis efficacy due to product accumulation. Equally, lab-produced cellulases show a low hydrolysis capability. *T. reesei* were selected in the study as they can secrete a variety of cellulase enzymes with different productivity, stability, specific activity, and synergism action [33]. Probably, our lab-produced cellulases were less stable or with less synergistic action inside the assembly of CF, resulting in a weaker hydrolysis action.

The cellulose OSHF process using the best performing CF (Sc/SG-cc) led to satisfactory ethanol production (6.3 mL/L) and cellulose conversion (61.2%). The results are similar to previous work [21]. Probably, a method to achieve a higher ethanol amount is to reduce the crystalline structure of cellulose [21].

Comparative performance of CF, GMO, and co-immobilization

The preparation of different CFs was conducted to ferment cellobiose. The CF of Sc/SG-cc achieved the best ethanol yield in cellobiose fermentation. CF of Sc/SG-cc produced higher cellobiose conversion and ethanol yield (82%) compared with results obtained in a process conducted by engineered *S. cerevisiae* or immobilized non-engineered *S. cerevisiae* with recombinant enzymes. Ethanol yields in the range of 70-73% with very low productivity (0.05-0.12 g/L/h) were obtained by engineered *S. cerevisiae* [12,34,35], and ethanol yield of 78% by non-engineered *S. cerevisiae* co-immobilized with recombinant enzyme [36].

The CF Sc/SG-cc was also used for cellulose OSHF with 6.3 mL/L ethanol yield, and the cellulose conversion reached 61.2%. The use of CF Sc/SG-cc produced higher ethanol concentration and yield compared with results obtained by engineered *S. cerevisiae, T. reesei,* and *S. cerevisiae* with pretreated cellulose, by consecutive hydrogenolysis, or by other pretreatments. Ethanol concentrations in the range of 3.8-5.4 g/L were obtained by engineered *S. cerevisiae* [37], 4.6-62.2% ethanol yield by consecutive hydrogenolysis [38] and 3.32-5.52 g/L ethanol concentration by other pretreatments [39].

Our study shows there is no necessity for recombinant yeast cells, and non-engineered yeast could be used in their natural form. Furthermore, the effectiveness and competitiveness of our CFs are worth consideration as a model for different appli-

cations in white biotechnology. That would simply require the substitution of *S. cerevisiae* with an appropriate microorganism for the desired product and enzymes according to the substrate being used.

Conclusion and future perspective

CF of Sc/SG-cc led OSHF resulting in 82% cellobiose conversion and 61.2% cellulose, Furthermore, CF of Sc-alu/SG-cc resulted in two-fold higher ethanol productivity than Sc/SG-cc. However, SG CF gave higher cellobiose conversion as compared with kis and alu CF. Comparatively, CF of Sc/SG-cc gave similar results for cellulose and cellobiose conversion, and ethanol yields, compared with those obtained by engineered *S. cerevisiae,* or co-immobilized non-engineered *S. cerevisiae* with recombinant enzymes. In the frame of it, the ongoing work will be focused on increasing ethanol yield and cellobiose and cellulose conversion by producing purer, more stable, and more synergistic active lab cellulases and reducing the crystallinity degree of cellulose. A techno-economic study will be conducted to find the cost-effective design of CF for OSHF of cellulose.

Declarations

CRediT authorship contribution statement: Kalogeropoulou: Methodology, Investigation, first draft; Plioni: Methodology, review & editing; Dimitrellou: review. Nigam: methodology, validation, editing; Kanellaki: Supervision; Koutinas: Conceptualization, Project administration, Funding acquisition.

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Abbreviations: CF: Cell factory; OSHF: One-step simultaneous hydrolysis and fermentation; GMO: Genetically modified organism; SG: Starch gel; Kis: Mineral Kissiris; alu: γ-alumina; Sc: *S. cerevisiae*; *Tr: Trichoderma reesei*; cc: Commercial cellulase; lc: lab-preparation of cellulases.

A-C: 9 designs of CF, each using 1 of 3 sources of cellulases:

[A]: CFs 1, 2, 3 without kis and alu

1. *S. cerevisiae*/starch gel-*T. reesei* (Sc/SG-Tr);
2. *S. cerevisiae*/starch gel-commercial cellulases (Sc/SG-cc);
3. *S. cerevisiae*/starch gel-lab produced cellulases (Sc/SG-lc);

[B]: CFs 4, 5, 6 using kis

4. *S. cerevisiae*-kissiris/starch gel-T. reesei (Sc-kis/SG-Tr);
5. *S. cerevisiae*-kissiris/starch gel-commercial cellulases (Sc-kis/SG-cc);
6. *S. cerevisiae*-kissiris/starch gel-lab produced cellulases (Sc-kis/SG-lc);

[C]: CFs 7, 8, 9 using alu

7. *S. cerevisiae*-γ alumina/starch gel-T. reesei (Sc-alu/SG-Tr);
8. *S. cerevisiae*-γ alumina/starch gel-commercial cellulases (Sc-alu/SG-cc);
9. *S. cerevisiae*-γ alumina/starch gel-lab produced cellulases (Sc-alu/SG-lc).

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