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**Ethanol from cellulose and cellobiose of woody-substrates in a single stage of 3-combined-bioprocesses employing a non-GM yeast cell-factory**

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## Abstract

The present study aims to prepare a *Saccharomyces cerevisiae* cell factory, to combine three bioprocesses in a single stage, including cellulolytic enzyme production, cellulose hydrolysis to glucose, and fermentation with low cost of nutrients to ethanol, without any genetic modification. Cell factories are prepared, in which *S. cerevisiae* cells are covered with starch gel (SG), either containing *Trichoderma reesei* (SG-*T. reesei*), or cellulase produced from *T. reesei* (SG-cellulase). The work comprises the cellulose bioconversion using *S. cerevisiae/*SG-cellulase, and *S. cerevisiae/*SG-*T. reesei* to carry out alcoholic fermentation in one batch with low nutrient cost. In addition, the effect of different concentrations of yeast in the cell factory on fermentation rate was examined. SEM, FTIR spectra analysis and cellulose fermentation were used to prove the successful preparation of cell factory and its activity to ferment cellulose in one combined bioprocess. The cellulose sourced from delignified sawdust was characterized with XRD spectra and porosimetry analysis. Cell factory models of *S. cerevisiae/*SG-*T. reesei* and *S. cerevisiae/*SG-cellulase were able to ferment cellulose from wood, obtaining 32% and 62% ethanol production yield, respectively. *S. cerevisiae*/SG-*T. reesei* and *S. cerevisiae*/SG-cellulase resulted to 4.07 and 7.30 mL ethanol/L, respectively. The final glucose concentration was very low (0-0.07 g glucose/L). It is also concluded that the increase of *S. cerevisiae* concentration in CF helped an improvement in the bioconversion process.

**Keywords**: cell factory; yeast; cellulase; cellulose; *Trichoderma*

## 1. Introduction

Cell factories in the frame of white biotechnology are crucial to producing products of industrial interest, mainly using renewable and low-cost raw materials such as lignocellulosic biomass (Hasunum & Kondo, 2012; Wang et al., 2018). Lignocellulosic biomass has been characterized as the second-generation raw material for fuel-grade alcohol production (da Silva et al., 2018; Naik et al., 2010). Generally, after completing the chemical pretreatment of plant biomass, the most effective and prospective methods for the hydrolysis of cellulose and cellobiose to glucose are employed, and then in the next stage, the alcoholic fermentation is carried out using yeasts to ferment sugars to ethanol (Liu et al., 2019; Sun & Cheng, 2002). However, using cellulase-producing microorganisms like *Trichoderma* is an additional process and therefore, the whole process needs three bioprocesses to be done separately to obtain the final product alcohol from polymeric substrates (Aditiya et al., 2016; Barrett et al., 1951). To achieve direct conversion of starch, efforts have been made for ethanol production by co-immobilized amyloglucosidase enzyme and *S. cerevisiae* (Chithra & Baradarajan, 1992). To avoid a three- or two-step bioprocess, molecular biologists tried to obtain a one-step process by genetic modification and engineering in plasmids of yeast cells, enabling a genetically-modified yeast to produce cellulase enzymes during saccharification of cellulose and cellobiose, and subsequent alcoholic fermentation (Liu et al., 2019; Liu et al., 2016). Though such expensive attempts have not yet resulted in any promising results (Liu et al., 2019). *S. cerevisiae* engineered to ferment xylose and glucose from hydrolyzed corn stalk using commercial enzymes, in simultaneous saccharification and co-fermentation resulted in 15 mL/L (11.84 g/L) ethanol (Zhao et al., 2019). Recombinant cellulolytic *S. cerevisiae* expressing

cellulase have been reviewed for ethanol production (1.25-10 mL/L, 0.99-7.89 g/L) from phosphoric acid swollen-cellulose (Oh & Jin, 2020). Hydrothermally processed rice straw with the addition of commercial cellulase and recombinant *S. cerevisiae* resulted in 17 mL/L (13.41 g/L) alcohol with the addition of nutrients (yeast extract, bacto-peptone) (Inokuma et al., 2014); After liquefaction of rice straw, the addition of nutrients, yeast extract and peptone, the higher ethanol yield was achieved 54 mL/L (42.60 g/L) (Matano et al., 2012). Similar concentrations of ethanol were obtained by simultaneous saccharification and fermentation (SSF) strategy (Wang et al., 2014).

Therefore, a study on bioconversion of cellulose sourced from wood is necessary and without nutrients using a new processing method based on submerged fermentation, due to several operating problems to perform SSF in a bioreactor design. Furthermore, a similar approach, two bioprocesses (alcoholic and malolactic fermentation) performed in the same batch, has been reported by Servetas et al. 2013, where a system of biocatalyst was designed with two layers of tubular cellulose (TC) and starch gel (SG) composite, containing a yeast strain *S. cerevisiae* in the internal surface of TC and a bacterial strain *Enoccocus oeni* immobilized on SG.

The proposed new technology involving the preparation of a cell factory of *S. cerevisiae* by covering its cells with SG which contained cellulase producer *T. reesei,* would be a practical model for white biotechnology. It is very important to examine if such a model cell factory can operate with low nutrient cost. The cell factory will allow three processes to occur simultaneously in one batch: (i) cellulolytic enzyme production, (ii) hydrolysis of cellulose to cellobiose, and cellobiose to glucose by cellulase enzymes produced by *T. reesei,* (iii) fermentation of glucose to ethanol by *S. cerevisiae*.

Therefore, the aim of this investigation is to produce a new generation cell factory without genetic modification of yeast cells, for a low-cost bioconversion of delignified wood sawdust. Three bioprocesses were combined in one stage with low nutrient cost, in order to reduce the investment and production costs. A second important goal of this cell factory is its presentation as a model, for the preparation of analogues cell factories in the frame of white biotechnology, using cellulose from bulk lignocellulosic raw materials to produce chemicals and value-added products.

## 2. Materials and methodology

### *2.1 Chemicals*

Chemicals used were - Starch (Penta, Czech Rep.); glucose, and ethanol (Fisher Scientific, UK); 2-propanol, $KH_2PO_4$, glycerol, and Tween-20 (Merck, Germany); potato dextrose agar (PDA), peptone, and soya peptone (Conda, Spain); NaOH (Lach-Ner, Czech Rep.); KBr, $(NH_4)_2SO_4$, and $MgSO_4.7H_2O$ (Chem-Lab, Belgium).

### *2.2 Microorganisms and media*

The baker's yeast *S. cerevisiae,* a commercial Lesaffre Hellas "L'hirondelle" product, was used for alcoholic fermentation. *T. reesei* (DSM No. 768, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) was used for cellulase enzymes production and for cellulose hydrolysis.

*T. reesei* was grown on PDA medium (agar, 15 g/L, dextrose, 20 g/L, potato extract, 4 g/L) at 30 °C for 6 days. After sporulation, the spores were collected from the surface of the dishes by aseptically scratching sporulated agar surface and washing with sterile distilled water (Liu et al., 2016). Spores' concentrations were determined using a haemocytometer (Neubauer Improved, HBG, Germany). All media were sterilized by autoclaving for 15 min (Plioni et al., 2022; Kalogeropoulou et al., 2022).

### *2.3 Delignification of wood for cellulose preparation*

Pinewood sawdust was obtained from a local timber industry. Chemical delignification was carried out by treating wood sawdust with a mild solution of NaOH (1%, w/v) for 3 h at 100 ºC. The ratio of wood sawdust/NaOH solution was taken at 1:10 (w/v), and the volume of the mixture was maintained constant during three hours of treatment by the addition of water (Iconomou et al., 1995; Plioni et al., 2022). The treated sawdust was washed thoroughly with hot water to remove the soluble lignin-sodium salt residues. Dried delignified sawdust was ground and sieved through a 0.1 cm sieve and stored at room temperature for further experiments as a source of cellulose in all experiments.

### *2.4 Preparation of cellulase enzymes*

*Seed culture*

*T. reesei* spores grown on PDA slants were washed with sterilized water to prepare a spore suspension of $10^7$–$10^8$ spores/mL. One mL of spore suspension was inoculated into 250-mL Erlenmeyer flasks, which contained 50 ml seed culture medium

(delignified sawdust 20 g/L, peptone 10 g/L, glucose 10 g/L, pH 4.5). Flasks were incubated for 24 h at 28 ºC with agitation at 180 rpm (Plioni et al., 2022; Kalogeropoulou et al., 2022).

*Cellulase production in the fermentor*

A 5%, v/v inoculum ($6x10^6$ spores/mL) of seed culture was inoculated into 2-L stirred fermentor (Electrolab Biotech Ltd.), using 1.0 L culture medium as working volume. The culture medium consisted of (g/L): cellulose (delignified sawdust) 50, soya peptone 17.0, $(NH_4)_2SO_4$ 5.0, $KH_2PO_4$ 6.0, $MgSO_4$ 1.0, glycerol (99% Sigma-Aldrich) 2.5, Tween-20 2 mL/L, pH 5.0. The culture conditions in stirred fermentor were maintained were: agitation speed 300 rpm, aeration rate 3 L/min, and temperature 26 °C. The culture filtrate (crude enzyme solution) was collected by centrifugation after 5 days of fermentation (Plioni et al., 2022; Kalogeropoulou et al., 2022).

*2.5 Preparation of cell factories*

*2.5.1 Preparation of S. cerevisiae/SG-T. reesei cell factory and cellulose fermentation*

Starch (puriss. p.a, Sigma-Aldrich) in a quantity of 16 g was added to 100 mL deionized water and heated at 90 ºC for 5 min, this starch gel (SG) was left to cool at room temperature. Subsequently, 10 mL of *T. reesei* spores ($5.3x10^6$ spores/mL) were mixed with the prepared SG. This preparation of SG/*T. reesei* biocatalyst was added dropwise on 6 g of fresh cell mass of baker's yeast, such prepared cell factory of *S. cerevisiae*/SG-*T. reesei* was freeze-dried. The dried cell factory was then used for

143 hydrolysis and alcoholic fermentation of 5g of cellulose (dried delignified sawdust) in
144 200 mL PD Broth. The hydrolysis and fermentation conditions were set at a
145 temperature of 30 ºC with gentle agitation (Plioni et al., 2022). Conversion-Yield was
146 calculated taking the cellulose content of softwood from the literature (Rowell et al.,
147 2012).

148

149 *2.5.2 Preparation of S. cerevisiae/SG-cellulases cell factory and cellulose*
150 *fermentation*

151 Starch in the quantity of 8 g was added in 100 mL deionized water and heated at 90
152 ºC for 5 min, this SG was left to cool at room temperature. 100 mL cellulase enzymes
153 preparation (397FPU) (described in section 2.4) was mixed with SG. Finally, the
154 SG/cellulase enzyme biocatalyst was added dropwise on 15 g fresh baker's yeast to
155 prepare the cell factory of *S. cerevisiae/*SG-*cellulases*. Then the whole mixture (200
156 mL) was used for hydrolysis and simultaneous alcoholic fermentation of 5g dried
157 delignified sawdust. The hydrolysis and fermentation conditions were set at a
158 temperature of 30 ºC with gentle agitation (Plioni et al., 2022). Conversion-Yield was
159 calculated by taking the value of cellulose content of softwood from the literature
160 (Rowell et al., 2012).

161

162 *2.5.3 Effect of concentration of S. cerevisiae/SG-T. reesei cell factory on cellulose*
163 *fermentation*

164 Starch (puriss. P.a, Sigma-Aldrich) in the quantity of 8 g was added to 100 mL
165 deionized water and heated at 90 ºC for 5 min, SG was left to cool at room
166 temperature. Subsequently, 30 mL of *T. reesei* spores ($5x10^6$, $17x10^6$ and $30x10^6$

spores/mL) were mixed with the prepared SG. Finally, the SG/*T. reesei* biocatalyst was added dropwise on 15, 20 and 25 g quantities of fresh cell mass of baker's yeast, and such prepared cell factory *S. cerevisiae/*SG-*T. reesei* was freeze-dried. The dried cell factory was then used for hydrolysis and alcoholic fermentation of 5 g dried delignified sawdust in 200 mL deionised water (Plioni et al., 2022). The hydrolysis and fermentation conditions were temperature 30 $^{o}$C and gentle agitation.

*2.6 Analyses*

*2.6.1 Determination of glucose and ethanol*

Hydrolysis and fermentation kinetics were performed by measuring glucose and ethanol concentration at various time intervals during a one-stage process. Shimadzu LC-9A HPLC system consisting of a Nucleogel Ion 300 OA column, an LC-9A pump, a RID-6A refractive index detector, a CTO-10A column oven, and a DGU-2A degassing unit, was used for the analysis of glucose and ethanol. An aqueous solution of 0.017 M $H_2SO_4$ was used as the mobile phase (flow 0.55 ml/min), with 1%, v/v 2-propanol as internal standard. The column temperature was set at 33 $^{o}$C, for sample dilution at 1%, v/v, and the injection volume was 40 μL (Kalogeropoulou et al., 2022).

*2.6.2 Scanning Electron Microscopy (SEM)*

The morphology of the dried biocatalysts (produced as described in Section 2.4) was observed by field emission SEM. All samples were coated with gold in a Balzers SCD

004 Sputter Coater for 10 min and examined in a JEOL model JSM-6300 scanning electron microscope (Barouni et al., 2015).

*2.6.3 Fourier-transform infrared (FTIR) spectroscopy*

FTIR spectrometer Perkin-Elmer (Waltman, MA, USA) was used in FTIR analysis for proving that no physical–chemical changes were conducted on starch. Starch, SG/*T. reesei* biocatalyst and *S. cerevisiae*/SG-*T. reesei* CF samples. Infrared spectra were recorded at 10 scans in the range 4000-300 $cm^{-1}$ with a resolution of 4 $cm^{-1}$. 2 mg dried samples were ground with 200 mg KBr. The whole mixture was pressed (8 tons for 5 min) for thin disc making (Drosos et al., 2021).

*2.6.4 X-ray Diffraction (XRD)*

The samples were dried in an oven (WT-Binder, max 100 °C). XRD Bruker (Billerica, MA, USA) D8 advance apparatus equipped with CuKa radiation as the incident light source Ni-filtered was used for obtaining XRD patterns. The intensity of diffracted radiation was measured between 5-60 $^{o}(2\theta)$ with a scanning rate of 0.1 $^{o}$/min (Barouni et al., 2015). The crystallinity index (CI) was calculated using the following equation (Gümüskaya et al., 2003):

$$CI(\%) = [(I_{max} - I_{min})/I_{max}]x100$$

where $I_{max}$ = strongest XRD peak intensity at $2\theta = 22$ and $I_{min}$ = background intensity at $2\theta = 18$.

*2.6.5 Textural Characteristics Determined by $N_2$ Adsorption/Desorption Experiments*

213 The specific surface area, the specific pore volume, and the pore size of the samples
214 were determined by measuring the amount of $N_2$ adsorbed on and desorbed over a
215 wide range of relative pressures. The $N_2$ adsorption-desorption experiments were
216 carried out at 77K using a Micromeritics apparatus (Tristar 3000 porosimeter). Before
217 the measurements, 0.4–0.6 g dried samples (oven WT-Binder, max 100 °C) were
218 degassed under vacuum at 110 ºC for 90 min (Barouni et al., 2015). The surface area
219 was calculated by the Brunauer–Emmett–Teller (BET) equation. The pore size of the
220 samples was estimated by the Barrett–Joyner–Halenda (BJH) method using the
221 desorption data (Barrett et al., 1951).

222

223 *2.6.6. Cellulase enzyme assay*

224 The sample was collected and centrifuged at 5000 rpm for 10 min. The supernatant
225 collected from centrifugation contains the enzyme. The supernatant was analyzed for
226 filter paper assay by the standard IUPAC method (Urbanszki et al., 2000; Siva et al.,
227 2022).

228

229 *2.6.7. Ethanol conversion calculation*

230 The delignified pine wood sawdust used in our experiments contains approximately
231 69% cellulose, 30% hemicellulose, and 1.5% lignin. The cellulose to glucose
232 conversion factor is 1.11 and the glucose to ethanol conversion factor is 0.51.

233

234 *2.6.8. Statistical analysis*

Duplicate experiments were conducted, and duplicate samples were used for the analyses. Experimental data were evaluated for their significance ($p < 0.05$, 0.01 and 0.001) with analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test using Statistica version 12.0 (StatSoft Inc., Tulsa, OK, USA).

## 3. Results and Discussion

### *3.1 Rational*

Simultaneous cellulase enzymes preparation, cellulose hydrolysis, and alcoholic fermentation in one batch without genetic modification of yeast cells have been attempted in the present investigation. This experimentation approach has been achieved through a new generation cell factory of *S. cerevisiae* without modification in the plasmid of yeast cells. The model of this cell factory was designed covering cells of *S. cerevisiae,* with the biopolymer starch in a gel form, which contained spores of *T. reesei*, producing cellulase enzymes under strong aeration and agitation creating aerobic conditions. The cellulolytic enzyme hydrolyzed cellulose and cellobiose to glucose, which could be fermented by *S. cerevisiae.* Therefore, all three bioprocesses could be performed in the same batch using the cell factory of *S. cerevisiae/*SG-*T. reesei.*

Furthermore, an alternative cell factory of *S. cerevisiae/*SG-cellulases was also prepared to ferment delignified sawdust material. It was prepared by the same method with the only difference of *T. reesei* being substituted with cellulase enzymes. Both versions of cell factories can be used as the model for the preparation of other cell factories to produce chemicals such as sweetener stevia, mannitol, flavour compounds

(Hugenholtz, 2008; Navarrete et al., 2020), in the frame of white biotechnology bioprocessing, without attempting the process of genetic modification. Due to the significance of this model cell factory, the focus of the investigation was the preparation of cell factories and demonstrating their operation in bioprocessing in simultaneous saccharification and fermentation of cellulose.

Due to the addition of nutrients in fermentation, the production cost is affected, mainly when the raw material used is obtained at low or free of cost. Therefore, it is important to examine if the cell factory can operate with low-cost nutrients. It is also of importance if two microorganisms are used for cellulose processing. The low nutrient cost was achieved by adding only potato extract in the case of delignified sawdust fermentation by *S. cerevisiae*/SG-*T. reesei,* and without nutrients addition in the fermentation by *S. cerevisiae*/SG-cellulase. The production of cellulase enzymes was done under strong aeration, while cellulose and cellobiose fermentation using a cell factory was performed under gentle agitation.

### *3.2 Preparation of cell factory of S. cerevisiae for cellulose and cellobiose fermentation in one batch*

The concept of cell factory is based on covering the yeast cells with a biopolymer such as SG, poly-ethylene-glycol (PEG), or alginates (ALG), containing either *T. reesei* or cellulase enzymes (Cacicedo et al., 2016). Fig. 1 SEM shows a layer of *T. reesei* cells without cells of *S. cerevisiae.* This supports the idea, that *T. reesei* cells are located on the upper level of that of *S. cerevisiae* cells. Fig. 2 illustrates FTIR spectra of starch, SG/*T. reesei* and of cell factory *S. cerevisiae*/SG-*T. reesei.* Similar absorptions for all samples were observed and no substantial differences could be

283 identified. Peak absorption at 1390 $cm^{-1}$ was attributed to the presence of carbonyl
284 groups in starch. The peaks at 3490 and 2940 $cm^{-1}$ are typical for O–H and C–H bond
285 vibrations. The absorption range corresponding to the hydroxyl group and the
286 hydrogen bond was observed at 3200–3500 $cm^{-1}$. Therefore, the similarity in all
287 spectra proves that *T. reesei* did not cause any change in the chemical structure of
288 starch. FTIR spectra in combination with SEM also proves the preparation and
289 composition of cell factory *S. cerevisiae*/SG-*T. reesei.*

290

291 *3.3 Delignified cellulosic (DC) material characterization*

292 The raw material used in our studies was delignified pinewood sawdust. Table 1
293 presents the textural characteristics of the delignified pinewood sawdust (cellulose) as
294 determined by $N_2$ sorption/desorption measurements. Comparing with samples from
295 different types of delignified cellulosic materials (mango 1.5 $m^2/g$; sal 0.6 $m^2/g$; rice
296 1.6 $m^2/g$; softwood 0.8 $m^2/g$; corn stalk 0.8 $m^2/g$; wheat straw 1.14 $m^2/g$) (Koutinas et
297 al., 2012; Kumar et al., 2014; Tsafrakidou et al., 2018; Peña-Gómez et al., 2020), it
298 can be observed that delignified sawdust cellulose has the same surface area as the
299 corn stalk, sal and softwood. The average pore diameter of nanotubes of the
300 delignified cellulose was 51.6 Å like observed in other studies.

301 XRD spectroscopy provided information on the crystalline structure of the studied
302 cellulose (delignified sawdust) of crystallinity index 60.8% and crystallite size 33.5
303 (Fig. 3). This characterization is done because the surface area of different
304 lignocellulosic materials differs substantially, as shown in the results presented above
305 for rice husk and sawdust (Wang et al., 2018). Likewise, the degree of crystallinity
306 also affects the rate of cellulose hydrolysis (Wang et al., 2018).

307

308 *3.4 Cellulose fermentation in one batch using cell factory without genetic*
309 *modification*

310

311 *3.4.1 Cell factory of S. cerevisiae/SG-T. reesei*

312 The fermentation was carried out in one batch under strong agitation of cellulosic
313 material to create aerobic conditions. Figure 4 illustrates the kinetics of alcoholic
314 fermentation and glucose concentration during the bioprocess. The fermentation was
315 completed in 3 days resulting in 4.07 mL ethanol/L (3.21 g/L) concentration with a
316 practical yield of 32%. The results prove the performance of the alcoholic
317 fermentation of cellulose in one batch using the cell factory of *S. cerevisiae*/SG-
318 *T.reesei* without genetic modification and with low-cost nutrients in the fermentation
319 of cellulose. The low nutrient cost was achieved by the addition of only potato extract.
320 During the bioprocess, glucose formation started at the beginning of the process and
321 reached its maximum concentration 8.0 g/L after 1 day and at this stage, the alcohol
322 concentration started to increase. At the end of the second day, the glucose
323 disappeared as all available glucose was converted to alcohol. To study in detail, the
324 effect of the microbiological composition of the cell factory, different cell
325 concentrations of *S. cerevisiae* and *T. reesei* were examined and results are presented
326 in Figure 5. It is indicated that the increase of yeast cell concentration increases the
327 rate of fermentation and therefore, the overall productivity is affected but it is not
328 statistically significant. The rate of fermentation was substantially increased when the
329 cell concentration was higher of $51x10^7$ cell/mL. Using this design of cell factory *S.*
330 *cerevisiae*/SG-*T.reesei*, almost 50% yield-conversion was obtained.

331

332 *3.4.2 Cell factory of S. cerevisiae/SG-cellulases*

333 The fermentation was carried out in one batch using the aforementioned cell factory
334 with the only difference that *T. reesei* was substituted with cellulase enzymes. Figure
335 6 illustrates the kinetics of delignified cellulose alcoholic fermentation and glucose
336 concentration during the fermentation. The fermentation of cellulose using *S.*
337 *cerevisiae*/SG-cellulase resulted in 62% yield conversion. Glucose during
338 fermentation was present in concentrations lower than 1.0 g/L. This result proved that
339 glucose released in the medium through the cellulolytic activity of cellulase from *T.*
340 *ressei* was immediately (simultaneously) fermented by *S. cerevisiae* to alcohol. The
341 fermentation of cellulose by *S. cerevisiae/*SG-cellulase was carried out without
342 nutrients. The results also show the cell factory of *S. cerevisiae*/SG-*T. reesei*
343 fermented cellulose with a lower rate than *S. cerevisiae*/SG-cellulase and with
344 reduced yield-conversion (Table 2).

345

346 *3.5 Scientific and Technological consideration of results*

347

348 The main objective of this investigation for the preparation of two types of cell
349 factories to ferment cellulose in one batch without genetic modification of yeast cells
350 and with low-cost nutrients. This objective has proved successful by analysis of SEM,
351 FTIR spectra and the results of a one-batch process combining simultaneous enzyme
352 production, cellulose saccharification and alcohol fermentation. This might lead to a
353 conceptual character of the research, following as model examples of two cell
354 factories of *S. cerevisiae*/SG-*T. reesei* and *S. cerevisiae*/SG-cellulase. Other types of

cell factories can also be designed using other microorganisms, to produce various value-added products using cellulose as the raw material. It opens a long way in the frame of white biotechnology to produce chemicals (Jami et al., 2010; Panagopoulos et al., 2022; Drosos et al., 2021) and foodstuffs (Hugenholtz, 2008; Navarrete al., 2020; Madzak et al., 2021).

In this area of designing new versions of cell factories, SG can be replaced by other biopolymers such as PEG and ALG (Cacicedo et al., 2016). Though the one-step cellulose fermentation was faster in the system of *S.cerevisiae*/SG-cellulase, the cell factory of *S. cerevisiae*/SG-*T. reesei* seems to be more applicable, due to one less step for cellulolytic enzyme production saving time and cost obtained with cheaper nutrients. The results presented in this study are a showcase of our efforts applied for the preparation of a cell factory, and to prove that this cell factory will operate with success for bioconversion of cellulose sourced from lignocellulosic materials. The results showed that the increase of yeast cell concentration in cell factories increased the rate of fermentation substantially. Our experiments in the ongoing project will be attempted to increase the alcohol yield and productivity.

The degree of crystallinity and crystallite aspect showed that the cell factories operated in cellulose fermentation in one batch, even for that crystallinity degree might affect the diffusion of enzymes into cellulose (Hall et al., 2010; Koutinas et al., 2016).

Considering the broad applications of this investigation, the economic impact of the results will be very positive. This can be attributed to the fact that cell factories of numerous microorganisms other than *S. cerevisiae* can be prepared, to bio-convert cellulose as raw material to other chemicals in one batch. Therefore, the main objective of this investigation was the preparation of cell factories and demonstrating

the operation using cellulosic biomass from wood as raw material with low-cost nutrients, which have been demonstrated by the outcomes of this study. Molecular biologists have not yet resulted in any promising results (Liu et al., 2019). *S. cerevisiae* engineered *employing* corn stalk using (Zhao et al., 2019). phosphoric acid swollen-cellulose (Oh & Jin, 2020), rice straw (Inokuma et al., 2014), resulted to 1.25-17mL/L (13.41g/L) ethanol. Therefore, wood has not examined as raw material and the characteristic of that bioprocess uses expensive nutrients which are not abundant and of high cost. Other strategies liquefaction of rice straw using nutrients (Matano et al., 2012) and solid-state fermentation (SSF) (Wang et al., 2014), even though achieve higher concentrations of ethanol have technical obstacles for the industrialization of the bioprocess. The main concept used in the preparation of this cell factory is the two microorganisms *S. cerevisiae* and *T. reesei* were enclosed or entrapped in separate layers, to avoid any contact between them. This strategy avoided biological competition between cells of different microorganisms. This is the necessity for the development of this cell factory. The SEM (Figure 1) proved that *T. reesei* was in the upper layer above the cells of *S. cerevisiae.*

*3.5.1 Consideration of Cell Factory cost*

Cell factory has two possibilities for its application. (1) First is the cell factory to be employed in repeated batches without making any change in it and then being discarded. (2) the cell factory was used in repeated batches with small changes made after the completion of the counts of *T. reesei*. Since in this case *S. cerevisiae* is covered and protected by SG/*S. cerevisiae* is an industrial microorganism able to perform repeated batches on an industrial scale. Therefore, the cell factory needs to make up the deficit of *T. reesei* counts after a substantial reduction of its activity in

405 repeated batches. This addition of *T. reesei* culture can be added in cell factory and
406 cells will be immobilized on starch by hydrogen bonding, Van Der Waals forces. We
407 take into account that the second possibility is not a good option, because the first one
408 does not need any additional work of making changes. In the second method, we need
409 in repeated batches to add an amount of *T. reesei* culture.

410 A bioreactor of 120,000 L is accompanied by a smaller vessel bioreactor of 20,000 L
411 from stainless steel for cell biomass production having a construction cost of about
412 €5,000. However, industrial bioreactors are accompanied by a bioreactor of this
413 capacity in traditional alcohol production plants. Considering that starch requirement
414 for the bioreactor of 120,000 L would be 10,000 kg costing about €7,000. Finally, this
415 can be paid one time for a long period, if at the end of the use of cell factory, the
416 starch of cell factory can be recovered by centrifugation, to prepare a new cell factory.
417 The aforementioned description shows relatively low investment mainly for the
418 bioreactors and starch. Therefore, this explains why the proposed cell factory is cost-
419 effective.

420

421 *3.5.2 Bio-conversion of cellulose*

422 Production of enzymes was obtained using *T. reesei* under strong aeration and cell
423 factory *S. cerevisiae*/SG-*T. reesei* was used in fermentation under mild agitation.
424 Under these conditions, *T. reesei* produces cellulase enzymes that hydrolyzes
425 cellulose (Saravanakumar & Kathiresan, 2014; Alfian et al., 2020). Therefore, glucose
426 was released from cellulose hydrolysis as presented in Figures 4 and 6. The yield is
427 calculated on cellulose content which does not differ substantially from conversion
428 (Poletto et al., 2010; Joshua et al., 2016). Table 2 shows kinetic parameters of
429 cellulose fermentation using non-GMO cell factories. The yield-Conversion has been

calculated considering cellulose content from the literature (Navarrete et al., 2020). The comparison of two cell factories is done in section 3.5.3 as below.

*3.5.3 Comparison of S. cerevisiae/SG-T. reesei with S. cerevisiae/SG-cellulases*

The results were obtained with 6g/200 mL *S. cerevisiae* in a cell factory of *S. cerevisiae/*SG-*T. reesei,* and with 15g *S. cerevisiae* in the cell factory of *S. cerevisiae/*SG-*cellulase (*Table 2). The yield-bioconversion of *S. cerevisiae/*SG-*T. reesei* was about 32% and lower than achieved with *S. cerevisiae/*SG-cellulase as 62 %. The rate of fermentation was also lower, due to the cell concentration of *S. cerevisiae* being much lower. Furthermore, cellulase has to be produced separately and therefore two bioprocesses were performed in the same batch, instead of three performed by *S. cerevisiae/*SG-*T. reesei.* To answer which bioprocess is of lower cost, we can stress that in the case of enzyme production in a second bioreactor, would need bigger factory and investment, more electricity for mass transfer and water requirements and increased labour cost. However, the safe answer about it could be obtained by a techno-economic analysis, even though the results are more promising for the cell factory *S. cerevisiae/*SG-*T. reesei.*

## 4. Conclusions

In the present study the cell factories of *S. cerevisiae*/SG-*T. reesei* and *S. cerevisiae*/SG-cellulase were successfully prepared and the alcoholic fermentation of cellobiose and cellulose was performed. In the fermentation of cellulose material from pinewood sawdust, 32% and 62% practical ethanol production yields were obtained,

454 respectively. The results showed that the rate of fermentation was substantially
455 increased when the concentration of *T. reesei* was higher of $51x10^7$ per mL. It is also
456 concluded that the increase in *S. cerevisiae* concentration resulted in an increase in
457 yield-bioconversion. This method produced satisfactorily results in 1-stage submerged
458 fermentation for bioconversion of cellulose from bulky wood biomass with low
459 nutrients cost, as compared with engineered *S. cerevisiae* operated with nutrients
460 yeast extract and peptone and with the addition of cellulase, in separate stages of
461 hydrolysis and fermentation of cellulose. The aforementioned cell factories act as
462 model systems in white biotechnology using different microorganisms other than *S.*
463 *cerevisiae,* to produce various products from cellulose in a one-batch process, thereby
464 completely avoiding the requirement of genetic modification of yeast.

465

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467

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474

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618 **LEGENDS**

619 **Table 1.** Porosimetry analysis of delignified wood cellulose (TC)

620 **Table 2.** Kinetic parameters of delignified wood cellulose on one-step hydrolysis-
621 fermentation at 30 °C using non-engineered *S. cerevisiae*/SG-*T. reesei* and non-
622 engineered *S. cerevisiae*/SG-cellulases CF

623 **Figure 1.** Scanning Electron Microscopy image of *T. reesei* in the upper layer of *S.*
624 *cerevisiae*/SG-*T. reesei* CF

625 **Figure 2.** FT-IR spectra of a) starch, b) SG/*T. reesei* biocatalyst and c) *S.*
626 *cerevisiae*/SG-*T. reesei* CF

627 **Figure 3.** XRD image of alkaline delignification of wood cellulose sample

628 **Figure 4.** Fermentation kinetics of 2.5% w/v dried delignified wood cellulose by *S.*
629 *cerevisiae*/SG-*T. reesei* CF prepared with 6 g non-engineered *S. cerevisiae* and
630 $5.3x10^7$ spores *T. reesei* on one-step hydrolysis-fermentatiomn at 30 °C without
631 nutrients

632 **Figure 5.** Fermentation kinetics of 2.5% w/v dried delignified wood cellulose by *S.*
633 *cerevisiae*/SG-*T. reesei* CFs prepared with different concentrations of non-engineered
634 *S. cerevisiae* and *T. reesei* on one-step hydrolysis-fermentation at 30 °C without
635 nutrients (low cells concentration: 15 g yeast and $15x10^7$ spores in CF; medium cells
636 concentration: 20 g yeast and $51x10^7$ spores in CF; high cells concentration: 25 g
637 yeast and $90x10^7$ spores in CF)

638 **Figure 6.** Fermentation kinetics of 2.5% w/v dried delignified wood cellulose by *S.*
639 *cerevisiae*/SG-cellulase CF prepared with 15 g non-engineered *S. cerevisiae* and 27
640 FPU/g cellulase on one-step hydrolysis-fermentation at 30 °C without nutrients

641 **Table 1**. Porosimetry analysis of delignified wood cellulose (TC)

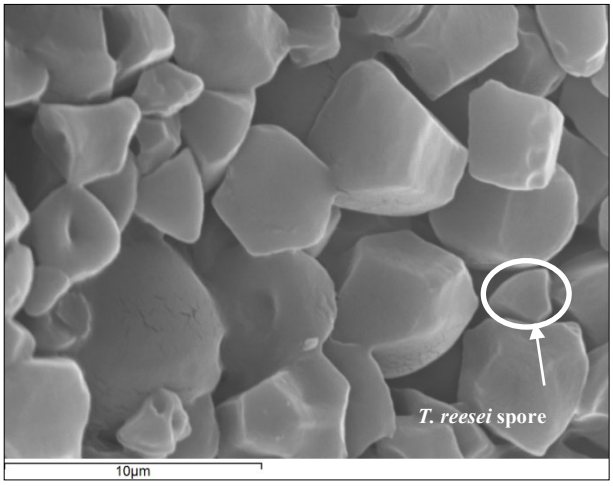
| | |
|---|---|
| Surface area ($m^2/g$) | 0.7131 |
| Pore size (Å) | 51.5558 |
| Pore volume ($cm^3/g$) | 0.000919 |

642 **Table 2.** Kinetic parameters of delignified wood cellulose on one-step hydrolysis-fermentation at 30 °C using non-engineered *S. cerevisiae*/SG-

643 *T. reesei* and non-engineered *S. cerevisiae*/SG-cellulases CF

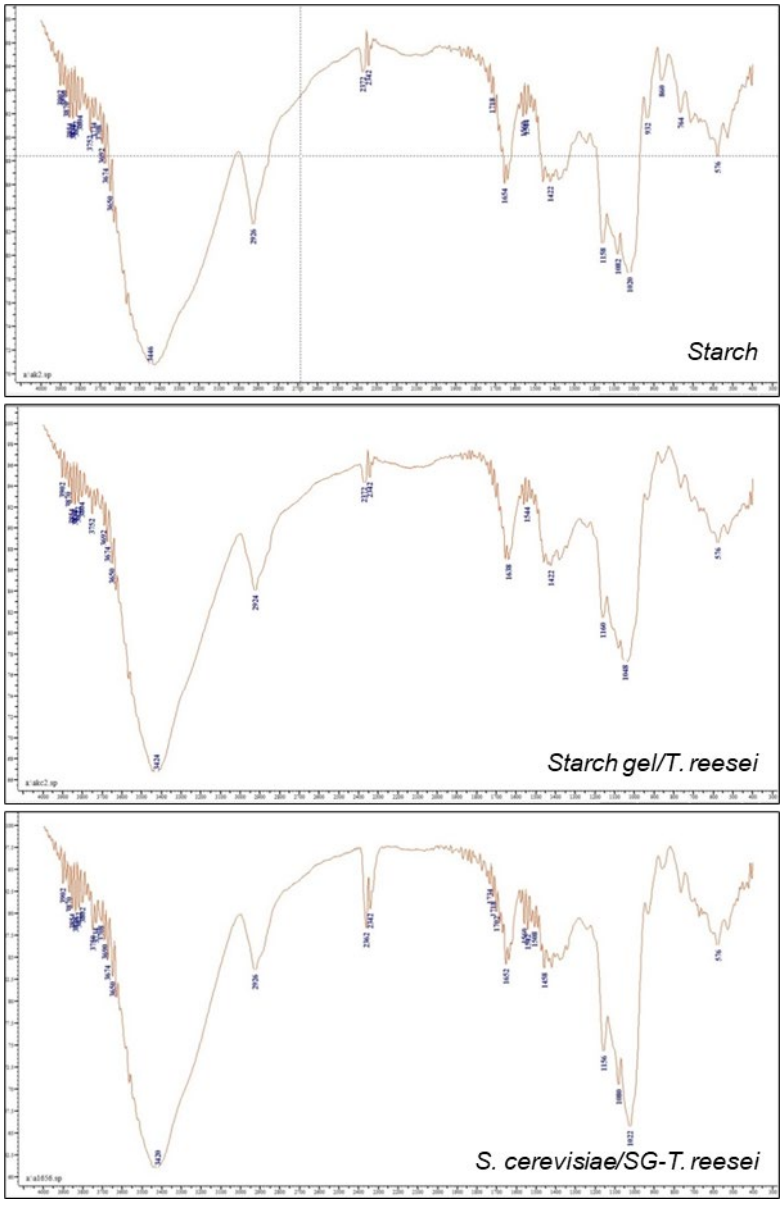
| Cell Factory | Initial delignified cellulose (g/200 mL) | *S. cerevisiae* concentration in CF (g wet weight cell/200 mL) | Fermentation time (h) | Maximum ethanol concentration | | Final glucose concentration (g/L) | Conversion (%) |
|---|---|---|---|---|---|---|---|
| | | | | (mL/L) | (g/L) | | |
| *S. cerevisiae* /SG-*T. reesei* | 5.0 | 6 | 72 | 4.07±0.13 | 3.21±0.10 | 0.0 | 32 |
| *S. cerevisiae* /SG-cellulases | 5.0 | 15 | 72 | 7.30±0.30 | 5.76±0.24 | 0.07±0.05 | 62 |

644




645 **Figure 1.** Scanning Electron Microscopy image of *T. reesei* in the upper layer of *S.*

646 *cerevisiae*/SG-*T. reesei* CF

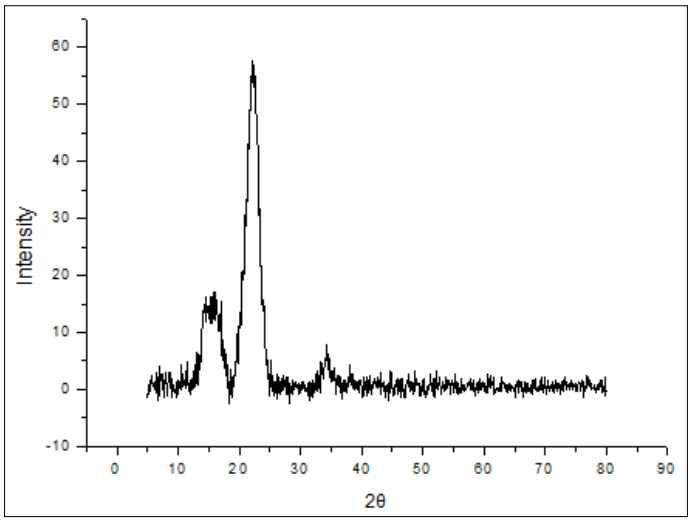



647

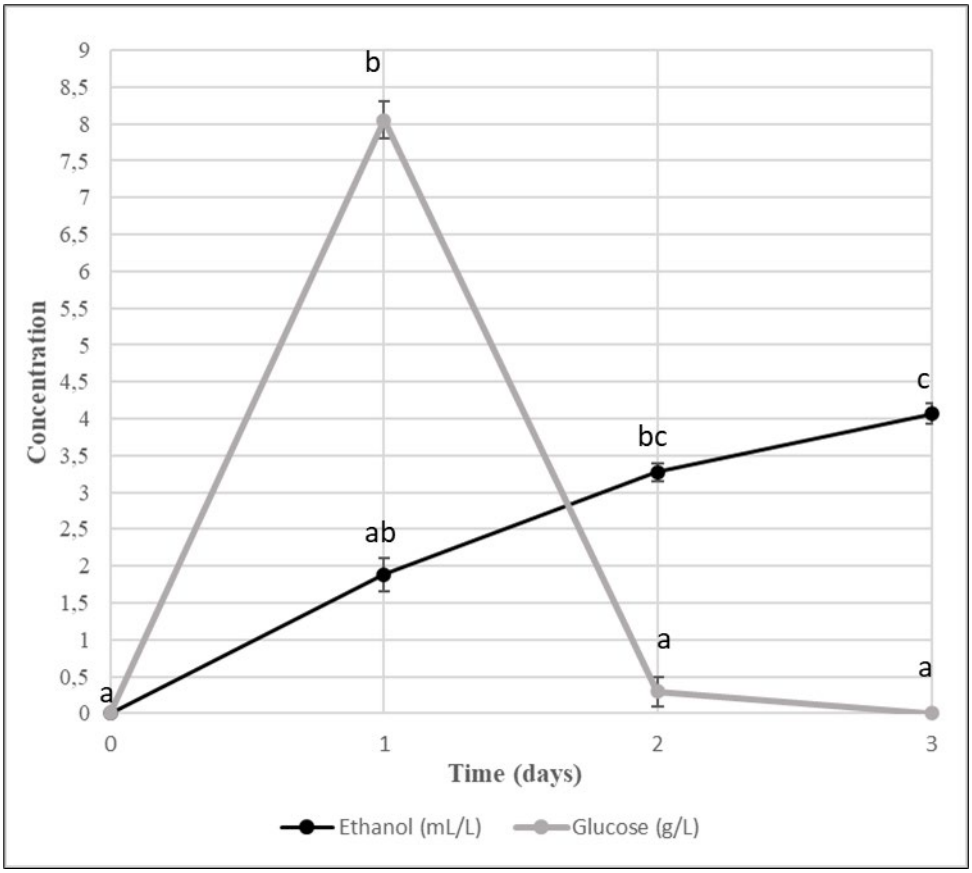
648 **Figure 2.** FT-IR spectra of a) starch, b) SG/*T. reesei* biocatalyst and c) *S.*

649 *cerevisiae*/SG-*T. reesei* CF

650




651 **Figure 3.** XRD image of alkaline delignification of wood cellulose sample




652

653 **Figure 4.** Fermentation kinetics of 2.5% w/v dried delignified wood cellulose by *S.*
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655 $5.3x10^7$ spores *T. reesei* on one-step hydrolysis-fermentation at 30 °C without
656 nutrients

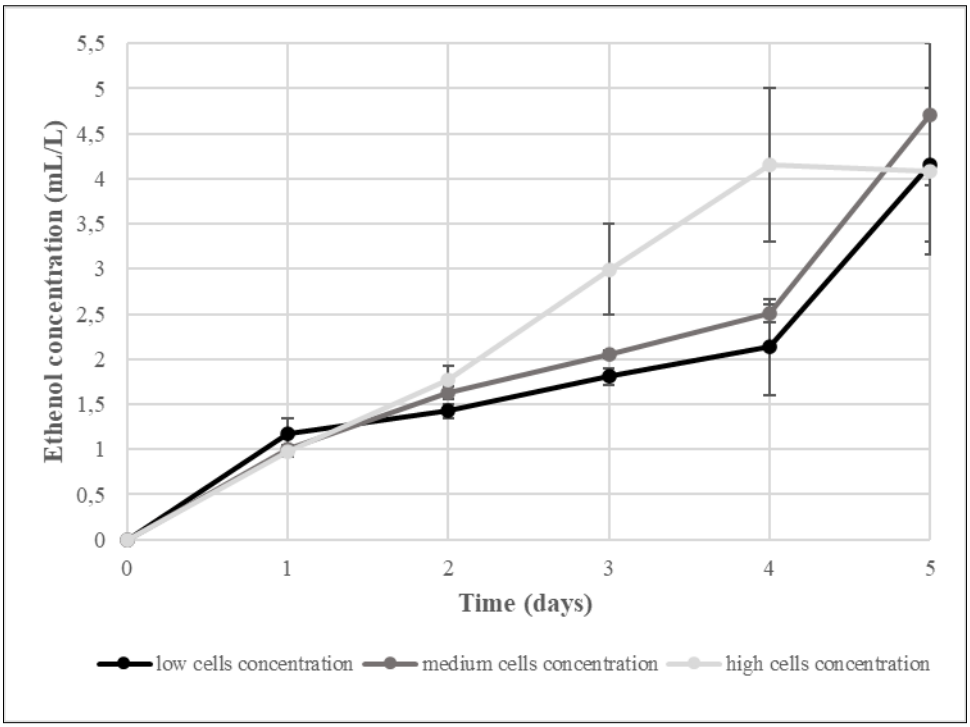


**Figure 5.** Fermentation kinetics of 2.5% w/v dried delignified wood cellulose by *S. cerevisiae*/SG-*T. reesei* CFs prepared with different concentrations of non-engineered *S. cerevisiae* and *T. reesei* on one-step hydrolysis-fermentation at 30 °C without nutrients (low cells concentration: 15 g yeast and $15x10^7$ spores in CF; medium cells concentration: 20 g yeast and $51x10^7$ spores in CF; high cells concentration: 25 g yeast and $90x10^7$ spores in CF)

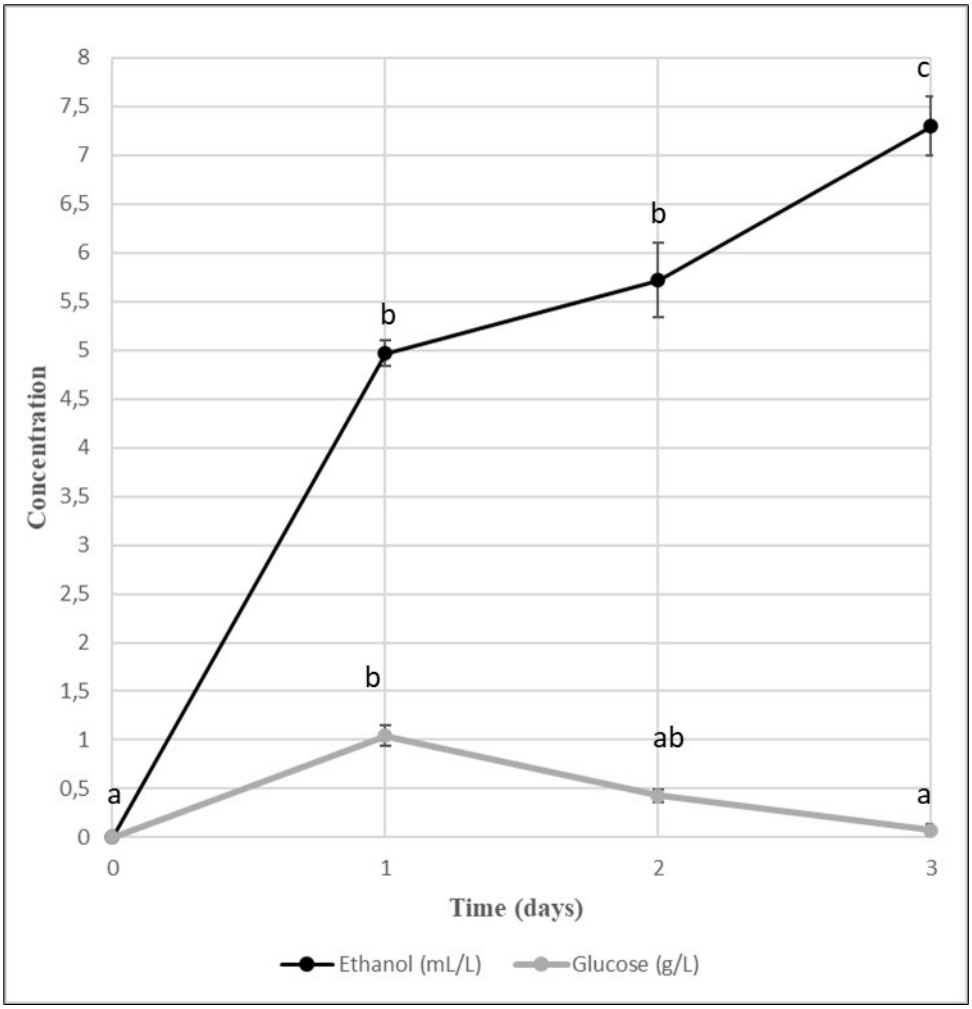


**Figure 6.** Fermentation kinetics of 2.5% w/v dried delignified wood cellulose by *S. cerevisiae*/SG-cellulase CF prepared with 15 g non-engineered *S. cerevisiae* and cellulase 27 FPU/g fresh baker's yeast on one-step hydrolysis-fermentation at 30 °C without nutrients