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Fermentation of Detoxified Acid-Hydrolyzed Pyrolytic Anhydrosugars into Bioethanol with *Saccharomyces cerevisiae* 2.399[1]

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Abstract—Pyrolysate obtained from the pyrolysis of waste cotton is a source of fermentable sugars that could be fermented into bioethanol fuel and other chemicals via microbial fermentation. However, pyrolysate is a complex mixture of fermentable and non-fermentable substrates causing inhibition of the microbial growth. The aim of this study was to detoxify the hydrolysate and then ferment it into bio-ethanol fuel in shake flasks and fermenter applying yeast strain *Saccharomyces cerevisiae* 2.399. Pyrolysate was hydrolyzed to glucose with 0.2 M sulfuric acid, neutralized with $Ba(OH)_2$ followed by treatment with ethyl acetate and activated carbon to remove fermentation inhibitors. The effect of various fermentation parameters such as inoculum concentration, pH and hydrolysate glucose was evaluated in shake flasks for optimum ethanol fermentation. With respect to inoculum concentration, 20% v/v inoculum i.e. $8.0 \times 10^8 - 1.2 \times 10^9$ cells/mL was the optimum level for producing 8.62 ± 0.33 g/L ethanol at 9 h of fermentation with a maximum yield of 0.46 g ethanol/g glucose. The optimum pH for hydrolysate glucose fermentation was found to be 6.0 that produced 8.57 ± 0.66 g/L ethanol. Maximum ethanol concentration, 14.78 g/L was obtained for 4% hydrolysate glucose concentration after 16 h of fermentation. Scale-up studies in stirred fermenter produced much higher productivity (1.32 g/L/h^{-1}) compared to shake flask fermentation (0.92 g/L/h^{-1}). The yield of ethanol reached a maximum of 91% and 89% of the theoretical yield of ethanol in shake flasks and fermenter, respectively. The complex of integrated models of development was applied, that has been successfully tested previously for the mathematical analysis of the fermentation processes.

Keywords: levoglucosan, bioethanol, hydrolysate, fermentation, *Saccharomyces cerevisiae*

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Climate change supposedly caused by global warming is a serious threat the world is facing, or going to face in the near future. The greenhouse gases (GHGs) are considered to be the main reason responsible for global warming [1]. About one-third of the GHGs are released from the transportation sector. In order to tackle the issue of climate change, GHGs emissions need to be reduced by replacing petroleum fuels with biofuel in the transportation sector [2]. However, China needs biofuel as an alternative fuel in its transportation sector, not only because of the widening gap between domestic crude oil supply and demand, but also to deal with the serious health risks associated with air pollution [3].

Bioethanol produced from sugar- and starch-based crops, or from the lignocellulosic biomass, represents a significant potential as a renewable biofuel that can not only be directly used as a liquid fuel, but also generally regarded as clean and environmentally friendly [3, 4]. The simple and readily convertible sugars present in sugary crops are easily and efficiently fermented into bioethanol by yeast and bacteria, however the food vs fuel debate makes it controversial. Ethanol produced from the inexpensive and abundantly available lignocellulosic biomass does not compete with food, however, the recalcitrant nature of the feedstock makes it quite a complicated and difficult process, multiple pretreatments are applied to the biomass material followed by hydrolysis requiring bulk quantities of acids and enzymes, and finally converted into ethanol via fermentation [3]. To overcome these bottlenecks, a relatively unexplored area of microbial fermentation of pyrolytic sugars, mainly levoglucosan and cellobiosan, is attracting much attention in recent times. These anhydrosugars have been detected in wildfire smoke at a concentration of 24 mg/g of organic carbon indicating that they are released into the atmosphere by forest fires or other typical biomass burning incidents, and later on recycled to the soil through rainwater and snow [5, 6]. Estimates show that out of the 4 billion metric tons of carbon

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released by biomass burning every year into the air, 90 million metric tons are anhydrosugars, indicating a significant portion of the under-characterized global carbon cycle [6].

These anhydrosugars are not only produced by the typical biomass burning incidents, but the process of fast pyrolysis - a controlled thermo-chemical destruction of the biomass material can also produce these sugars [7]. The advantages of the fast pyrolysis process are that it overcomes the recalcitrant nature of the lignocellulosic biomass by eliminating the complex pretreatment steps, and the requirement for costly enzymatic treatment. Further, it directly converts the whole biomass into fermentable intermediates irrespective of the type and composition of the feedstock material [8]. The process of fast pyrolysis results in the production of a dark brown or dark green liquid substance known as pyrolysate, pyrolysis oil or bio-oil, which is a complex mixture of more than 400 compounds. The rich chemical composition of the bio-oil makes it a source for the platform chemicals (glyceraldehydes, acetic acid and acetol) as well as fermentable substrates such as levoglucosan, cellobiosan, acetic acid, glyceraldehydes and hydroxyacetone that could be converted into ethanol, lipids, and other useful chemicals via microbial fermentation [3]. Major anhydrosugar in pyrolysis oil is levoglucosan, and its yield can be as high as 60 wt % based on cellulose concentration [9].

Direct microbial utilization of levoglucosan has been reported via levoglucosan kinase in filamentous fungi and yeast strains as well as levoglucosan dehydrogenase in bacterium *Arthrobacter* [10–14]. However, most of these utilizers do not produce ethanol from levoglucosan, or even if some produce ethanol, the yield is quite low. In order to ferment the bio-oil containing levoglucosan, acid-hydrolysis is applied to convert levoglucosan into glucose, which is later fermented by yeast and bacteria to produce ethanol and lipids [3]. Previously, some studies have been conducted to produce bioethanol from the acid-hydrolyzed bio-oil [4, 15–21]. Though all the previous studies regarding bio-ethanol production from acid-hydrolyzed bio-oil are promising, toxicity posed by the inhibitors present in bio-oil still remains a big challenge. The process of detoxification determines whether a toxic hydrolysate could be fermented into ethanol or not. However, the overall yield and productivity basically depend on the selection of robust microbial strains as well as the optimization of the fermentation parameters for ethanol fermentation [4]. *Saccharomyces cerevisiae* 2.399 was selected as the fermentative strain in this study, based on its efficient bioconversion of acid-hydrolyzed bio-oil to ethanol, compared to *Zymomonas mobilis* 10232, and *Pichia* sp. YZ-1 in previous studies by our group [15]. High ethanol yield in relatively short fermentation time is very important for the economic viability of pyrolysis-based biorefineries, and this in turn depends on inoculum concentration, pH, substrate (glucose concentration), along with other factors affecting microbial growth and metabolism. Most of the previous studies regarding hydrolysate fermentation have been carried out in shake flasks. Scale-up studies in the fermenter are very crucial to the commercialization of pyrolysis-based bio-refineries. The aim of the present study was to improve the fermentability of the hydrolysate glucose by applying detoxification strategies, to increase the overall yield and productivity of ethanol via process optimization in shake flask and scale-up studies in the fermenter using *S. cerevisiae* 2.399.

MATERIALS AND METHODS

Pyrolysis oil. Pyrolysis oil used in this study was prepared from untreated waste cotton according to the method of Zhuang et al. [10] and stored in airtight bottles at 4°C. According to our previous paper the main anhydrosugar in the pyrolysate was levoglucosan (146.36 g/L) [4].

Hydrolysis. Prior to hydrolysis, the pyrolysis oil was 3-fold diluted with water, kept on a shaker at 100 rpm for 10–20 min to achieve complete mixing, and then centrifuged at 3000× *g* for 15 min. The sugar-containing supernatant was collected in separate Falcon tubes, while the pellet was discarded. Acid hydrolysis was applied to convert the pyrolytic anyhydrosugars present in the pyrolysate to fermentable glucose. Hydrolysis was carried out according to the previously optimized method by Yu and Zhang [15] for the cotton pyrolysate. Sulfuric acid was added to the diluted pyrolysate to a final concentration of 0.2 M, and then autoclaved for 20 min at 121°C. The acid-hydrolyzed pyrolysate was neutralized to a pH around 6.0 with $Ba(OH)_2$, and then filtered through a filter paper (Whatman, Sigma-Aldrich, USA) to remove the precipitate.

Detoxification. Pyrolysate was extracted with ethyl acetate according to the optimized ratio of pyrolysate to ethyl acetate 1 : 2 (vol/vol) [4], one part of the pyrolysate was mixed with 2 parts of ethyl acetate, and then kept on a shaker for 4–6 hours at 150 rpm and 30°C. In order to achieve complete phase separation, the mixture was left to stand for 30 min at room temperature. The upper organic layer was removed by pipette, and the remaining dissolved ethyl acetate was evaporated at 50°C in an oven. The hydrolyzed pyrolysate was further detoxified with adsorption on 5% (wt/vol) activated carbon; the resulting slurry was kept for 6 h at 25°C in a rotary shaker at 100 rpm. Upon completion, the hydrolysate was filtered through a filter paper (Whatman, Sigma-Aldrich, USA) to remove the activated carbon residues.

Microorganisms and fermentation media. The detoxified hydrolysate was subjected to fermentation using yeast strain, *S. cerevisiae* 2.399, which was initially obtained from China General Microbiological Culture Collection Center (CGMCC, China). It was

maintained on a medium containing (g/L): yeast extract – 10.0, peptone – 20.0 and glucose – 20.0 (at 4°C), and subcultured every month at 30°C. The inoculum was prepared from the slant cultures in 250 mL Erlenmeyer flasks containing 100 mL yeast extract peptone dextrose (YPD) medium (Sigma-Aldrich, USA) grown on a rotary shaker at 30°C and 150 rpm for 24 h. Then, 2 mL of the starter culture was transferred to a fresh YPD medium and grown for another 24 h and later on used for subsequent inoculations. The yeast concentration in the inoculum culture was approximately 4×10^7–6×10^7 cells/mL. The initial fermentation optimization experiments were also conducted in 250-mL Erlenmeyer flasks containing 100 mL medium. Batch fermentation experiments were carried out in a fully automated, 7-L stirred fermenter (BIOTECH-5TBS, Shanghai Baoxing Bio-Engineering Co., Ltd, China). All the probes were connected to the proportion integration and differential controller in order to control the process automatically. Sterile air for fermentation was provided by a compressor equipped with a 0.22-μm filter; media sterilization was achieved through a steam generator. Batch fermentation was carried out with a 3-L working volume. The medium used for the fermentation experiments in the fermenter and shake flask was the same as the inoculum preparation medium, however the pure glucose was replaced with the hydrolysate glucose. The fermentation medium was *in situ* autoclaved in the fermenter at 115°C for 20 min. The temperature was set at 30°C, while the pH was maintained at 6.0 by the automatic addition of 3 M NaOH. Agitation speed was set at 150 rpm, while aeration was controlled at 2.5 vvm for the first 4 h.

Mathematical Model

Basic assumptions of the proposed integrated model. The proposed integrated model of development (IMMD) was previously successfully applied to describe growth dynamics of unicellular microorganism populations in periodic and continuous processes, and demonstrated its high adequacy [22, 23]. The model integrates 3 key components.

(a) The unstructured model previously developed by Derbyshev et al. [24] allows calculating energy costs for growth and vital activity of population units, total maximum quantity of units accumulated in separate population and other parameters.

(b) The structured growth model just allows calculating the structure of a growing population divided into 2 parts, including stable (resting) cells (stage B of cell cycle in prokaryotes, stages G0 and G1 in eukaryotes) and dividing (developing) cells at any stage of the cell cycle except for the stages mentioned above. The main indicator of population structuring is determined as a ratio of the number of stable (resting) population units to the total number of population units. Such a model enables one to determine the rate of stable cells accumulation, and accordingly, time of cessation of all cell divisions, which is the final time of development under specific conditions [22, 23].

(c) The model of metabolism allows calculating both dynamics of source nutritional elements consumption and dynamics of metabolites formation that are produced during life activity, and in turn may be used as nutritional elements under certain conditions. Therefore, the model of metabolism for source nutritional elements and derived products is described by the same equation. Unstructured and structured models of growth dynamics of biological objects- biomass (1) and also the model of their metabolite production or substrate consumption (2) have the following form [22, 23].

$$\frac{d^n X^{\mathrm{div}}}{d(X^{\mathrm{st}})^n} = \frac{K}{A^2}\frac{(-1)^{(n-1)}n!}{(X^{\mathrm{st}})^{(n+1)}} - C. \quad (1)$$

The equation of a structured model for substrate (metabolites) in growth inhibition phase (GIP) is

$$d(P \text{ or } -S)/d\tau = k^{\mathrm{div}}_{P,S} X^{\mathrm{div}} + k^{\mathrm{st}}_{P,S} X^{\mathrm{st}}, \quad (2)$$

where n is the integer number that determines derivative order of this function; X^{div} is the number of dividing cells; X^{st} is the number of resting (stable) cells; P is the target metabolite product; S is a substrate; constant K is a product of total growth rate and the rate of stable (resting) cells accumulation; A is the ratio of energy for maintenance of life activity to energy consumed for biomass growth and/or the rate of resting cells accumulation, $k^{\mathrm{div}}_{P,S}$ and $k^{\mathrm{st}}_{P,S}$ are constants. In addition, $C = 1$ if $n = 1$, and $C = 0$ if $n \geq 2$.

Parameter A, which was not used in any practical way earlier, estimates periodic culture growth and describes a delay of total biomass growth rate. Delay of growth during GIP occurs because of a presence of limitation factors such as low concentrations of dissolved oxygen, nutrients, medium heterogeneity, or other variables that provoke growth arrest. During GIP, the share of stable cells within the population is equal to that of non-proliferating cells, which consume energy. At that point, accumulation of stable cells occurs at a constant specific rate equal to that of the growth delay ($A = m/a$), where "m" is a maintenance energy and "a" is a tropic coefficient. On the basis of the present model, we described consumption of substrates used for cell construction and synthesis of metabolites in the cultures consisting of 2 groups of cells different in energy consumption. Specific growth rate of biomass X per $h(\mu)$, maximum culture specific growth rate ($\mu_{\max}$), and other significant parameters were calculated according to methods described previously [22, 23].

The main idea of the models lies in the following. It is assumed, that metabolites are synthesized only by proliferating cells. Non-proliferating cells, as a rule, destroy these products. Therefore, signs of the constants k^{st} and k^{div} for metabolite synthesis and degrada-

tion are opposite. The same should be stated for substrates utilized for cell construction. We always see the product as a result of only the "positive" with k^{div} constant synthesis processes, but it is known as "negative" with k^{st} constant processes also always occur. It is the destruction of the product, preventing its syntheses with any unknown reasons, etc. The same can be said for the utilization of substrates.

Features of working with the model. Features of working with the model have been described in detail in our previous articles [22, 23].

(a) For biomass:

To define the parameters of the model, we must take samples of fermentation culture through the same time periods, $\Delta\tau$. Then we build dependencies $\Delta X = f(X)$. For the logarithmic growth phase (LGP) we will have straight lines that begin at the point, (0; 0). The values of these functions are increasing for LGP with increase of X-values up to the value X_{Lim}, and then they begin to fall according to another straight line for a slow growth phase, GIP. For the analysis of biomass growth, the following parameters are determined first: biomass concentration, X_{Lim}, and time, τLim, corresponding to LGP termination and beginning of GIP; hypothetical maximum biomass concentration, X_p, when all energy transformed by the system is consumed for biomass viability maintenance; A = m/a is the specific growth delay rate of the biomass during GIP. Where A-value is the value of the angle for line $\Delta X = f(X)$ in the slow phase, GIP, which was converted on certain equations of the model. In addition, A-value is a specific rate of accumulation of stable cells [23, 24]. Parameters of the unstructured model τ_{Lim}, X_{Lim}, X_p depend on the selected oxygen mass exchange. All the parameters depend on nutrient medium composition and cultivation conditions. Then all the parameters of the unstructured model are used for calculating the parameters of the structured model and biosynthesis constants. In the work given, μ_{max} was calculated by a standard technique [22–24]. In this case, dependence of LGP natural logarithms, LnX, on time τ was built and the tangent of the inclination angle of the obtained straight line was determined. These values were compared to the results obtained with model equation (37) described in article of Derbyshev et al. [24].

(b) For products and substrates.

If we divide both parts of the Eq. (2) for X, we will get the following equation:

$$q = k^{div} + (k^{div} - k^{st})R, \quad (3)$$

where R is a ratio of X^{st} to biomass X, relative content of stable cells, X^{st}, in a total biomass, X.

If we defined previously parameters of a total biomass, then we can find R and q values for each point of time to build a schedule for Eq. (3). On this schedule we will be able to define the parameters for the products/substrates.

Analytical Methods

Quantification of the different compounds present in the pyrolysate, hydrolyzed pyrolysate, hydrolyzed-neutralized pyrolysate, and the hydrolysate extracted with ethyl acetate was conducted with an Agilent 6890N gas chromatography system (USA). The GC-MS system was connected with a mass spectrometry detector provided with a capillary column (CNW CD-5MS, Agilent Technologies, USA). Samples were prepared with methanol containing 5% pyrolysate or treated hydrolysate. Prior to injection, 1 μL samples were passed through 0.45 μm micro-filters, (Agilent Technologies, USA) and then injected into the injection port set at 200°C with a split ratio of 10 : 1. The column was run at a constant flow mode using helium as the carrier gas at a flow rate of 1 mL/min. Initially, the column was held at 40°C for one min, and then heated to 280°C with heating rate of 3°C/min, and finally held at this temperature for 10 min. The mass spectrometer was operated with an electron ionization temperature set at 70 eV, and transfer line temperature and an ion source temperature of 230°C. The masses of the different samples obtained were scanned from 28 to 400 amu. The different compounds present in the pyrolysate and treated hydrolysate were identified on the basis of their retention time, and comparison with the mass spectra values recorded with those reported in NIST mass spectral database. Due to the complex chemical nature of the pyrolysate, and the unavailability of the commercial standards for the compounds present in the pyrolysate, the GC-MS could not quantify all the compounds present in the pyrolysate. Therefore, compounds such as formic acid, acetic acid, furfural, and 5-hydroxymethylfurfural (5-HMF) that are important from the perspective of microbial growth inhibition were selected for quantification. For other compounds, their chromatographic peak area values for different samples were compared in order to estimate changes in their relative concentration.

For residual glucose, ethanol, and dry cell weight estimation, about 4 mL samples were taken at 3 h of fermentation for the shake flask and at 2 h for the fermenter. Samples were centrifuged at 8000× g and 4°C for 10 min, pellets containing cells and media components were washed with distilled water, and kept in an oven at 65°C for dry cell weight estimation. The supernatant was used for ethanol and glucose determination. Glucose and ethanol in fermentation samples were detected by HPLC system (LC-20AT, Shimadzu Corporation, Japan) equipped with a RID-10A refractive index detector, and a HCT-360 column heater. The column used was a Transgenomic ICSep ICE-ION-300 column (7.8 × 300 mm; Transgenomic Inc., USA). Sulfuric acid (0.00085 N) was used as a mobile phase at a flow rate of 0.4 mL/min with a sample injection volume of 20 μL. The column temperature was maintained at 58°C. In each case, 3 replicates

Table 1. GC-MS analysis of the pyrolysate, hydrolysate, hydrolyzed-neutralized pyrolysate, and pyrolysate after extraction with ethyl acetate

Compound in pyrolysate	Percentage of residual to original concentration, %			
	pyrolysate, g/L	hydrolyzed pyrolysate	hydrolyzed-neutralized pyrolysate	after ethyl acetate extraction
Formic acid	10.02	100	8.2	<0.1
Acetic acid	46.32	100	15.8	<0.1
Furfural	12.72	25	<0.1	<0.1
5-Hydroxymethylfurfural	37.20	11.4	3.1	<0.1
1,6-Anhydro-β-d-glucopyranose (levoglucosan)	146.36	<0.1	<0.1	<0.1

were analyzed. All the reagents used in this study were of analytical grade.

RESULTS AND DISCUSSING

Hydrolysis and Detoxification of the Pyrolysate

HPLC analysis of the crude pyrolysate showed that levoglucosan was the predominant anhydrosugar (146.36 g/L); however after acid-hydrolysis, the levoglucosan peak completely disappeared from the chromatogram, while glucose peak became visible indicating that all the levoglucosan was hydrolyzed to glucose. The glucose concentration reached up to 216.68 g/L, exceeding the theoretical yield of glucose, based on the original concentration of levoglucosan. The same observation was initially observed by Yu and Zhang [15, 16] and later by other researchers [4, 17, 20]. All these authors explained the increase in glucose concentration beyond the theoretical yield by the fact that cellobiosan, and other unknown carbohydrate oligomers are also converted to glucose during acid-hydrolysis, resulting in an overall increase in glucose concentration.

Table 1 shows the GC-MS analysis of the pyrolysate, hydrolyzed pyrolysate, hydrolyzed-neutralized pyrolysate, and hydrolysate extracted with ethyl acetate using the ratio of the pyrolysate to ethyl acetate 1 : 2 (vol/vol). The GC-MS analysis revealed 1,6-anhydro-β-D-glucopyranose (levoglucosan), 1,4:3,6-dianhydro-α-D-glucopyranose, 1,6-anhydro-α-D-galactofuranose, acetic acid, formic acid, *n*-hexane, maltol, acetol, furfural, 2(5H)-furanone, 5-HMF, glyceraldehyde, and *cis*-1,2-cyclohexanediol in the pyrolysate samples. Although crude pyrolysate is a promising source of fermentable substrates, such as levoglucosan, cellobiosan, acetic acid, glycoaldehyde, and hydroxyacetone, however, it also contains compounds that are toxic to microbial growth. Studies have reported that not only the non-fermentable substrates, but also the fermentable ones, like acetic acid can pose toxicity to the growth of microorganisms [3]. Previous research has proved that acetic, formic and levulinic acids, furfural, and 5-HMF are inhibitory to yeast fermentation [3, 25]. These compounds were selected for GC-MS quantification. The GC-MS quantification showed that the pyrolysate contained (g/L): acetic acid—46.20, formic acid—10.10, furfural—12.52, and 5-HMF—37.04. As shown in Table 1, the concentration of many compounds significantly decreased after hydrolysis, it might be due to the char-like precipitate formation that was observed each time after pyrolysate hydrolysis. Levoglucosan, 1,4:3,6-dianhydro-α-D-glucopyranose, and 1,6-anhydro-α-D-galactofuranose were not detected at all, while the concentration of furfural and 5-HMF reduced to significant levels. After hydrolysis, the hydrolysate was neutralized with $Ba(OH)_2$ and GC-MS analysis revealed that the concentration of most of the compounds, such as propionic and formic acids, acetol, furfural, maltol, 5-HMF, *n*-hexane, and methyl acetate, decreased to significant levels after neutralization, while concentrations of acetic acid, formic acid, and 5-HMF dropped to almost zero, while furfural was not detectable. However, even then, the yeast could hardly grow in the hydrolysate, indicating that the hydrolysate still contained some unknown inhibitors, or possibly some unknown additional inhibitory compounds were produced during the acid-hydrolysis step at high temperature and pressure that were not detected by the GC-MS.

Therefore, in order to let the yeast grow and ferment the hydrolysate glucose, detoxification was necessary in order to remove the toxic compounds such as phenolics, furans, and aldehydes. We applied a combination of detoxification strategy involving fractionation of the pyrolysate using solvent extraction followed by adsorption on activated carbon. Ethyl acetate was the solvent of choice based on its effective detoxification of phenolic compounds and acetic acid reported in previous studies [4, 17, 19, 21]. In our study, we applied an already optimized ratio of the hydrolyzed pyrolysate to ethyl acetate (1 : 2 vol/vol) detected in our previous paper [4]. After applying the ethyl acetate treatment, samples of the hydrolysate were analyzed on GC-MS, which showed that the levels of acetic acid and 5-HMF dropped to almost zero,

while formic acid was not detected at all as shown in Table 1. As previously mentioned, the pyrolysis oil is chemically very complex, we assume that there might have been other toxic compounds that were not detected by GC-MS, but they might have an inhibitory effect; further, the acid-hydrolysis is thought to introduce additional inhibitors. It is also important to mention that the inhibitory effect of toxic compounds is more severe when they coexist in the same system [26]. Previous studies have reported that adsorption on activated carbon in combination with other detoxification methods, resulted in overall improvement in fermentation [15, 17, 18, 27]. In order to grow the yeast efficiently and produce higher yields of bioethanol, we treated the hydrolysate with 5% (wt/vol) activated carbon though the previous studies of Yu and Zhang [15] and Wang et al. [18] reported some loss of sugars following activated carbon adsorption. However, no loss of fermentable sugars were observed in our study, which might be attributed to the fact that the dosage of activated carbon and treatment conditions applied in this study were different to those used in previous studies. Yu and Zhang [15] used 10% (wt/vol) activated carbon after neutralization or over-liming with $Ca(OH)_2$. Though they reported the loss of sugars, but did not explain whether the loss of sugar was due to neutralization/over-liming or activated carbon. Further, the dosage of activated carbon they applied was double compared to this study.

Hydrolysate Fermentation in Shake Flasks

Effect of inoculum concentration on hydrolysate fermentation. The size of inoculum is one of the key process parameters in microbial fermentations [28]. Different concentrations of the inoculum ranging from 1 to 30% (vol/vol) were tested in shake flasks in order to evaluate the effect of the increase in cell density on bioethanol fermentation of the hydrolysate glucose. Figure 1 shows the effect of different concentrations of inoculum after 3, 9 and 12 h of fermentation. Figure 1a explains that at the start of fermentation, the growth of the yeast was very slow for the small inoculum concentrations (1%; 4×10^7–6×10^7 cells/mL and 7%; 2.8×10^8–4.2×10^8 cells/mL) as compared to the higher inoculum density. However, the yeast started to grow faster, the level of hydrolysate glucose also started to drop, and even some ethanol was produced for the medium size inocula (15%; 6.0×10^8–9.0×10^8 cells/mL and 20%; 8.0×10^8–1.2×10^9 cells/mL). On the other hand, due to the high number of yeast cells in 25% (1×10^9–1.5×10^9 cells/mL and 30% (1.2×10^9–1.8×10^9 cells/mL) inocula, the process of fermentation was very rapid, the hydrolysate glucose was almost completely utilized in the first three h of fermentation, and also produced better ethanol titers i.e. 4.57 and 6.39 g/L, respectively. Table 2 shows that 25 and 30% inocula consumed all the hydrolysate glucose after 6 h of fermentation, and

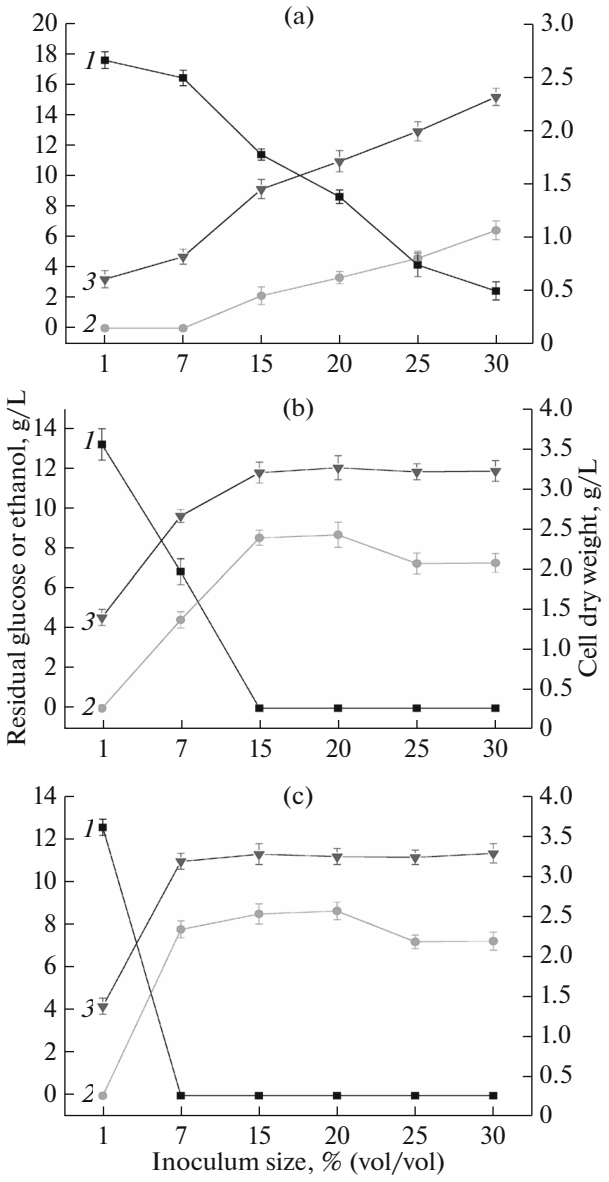

Fig. 1. Effect of the *S. cerevisiae* 2.399 inoculum size on ethanol fermentation of detoxified hydrolysate glucose after 3 (a), 9 (b) and 12 h (c). (*1*) Residual glucose; (*2*) ethanol; (*3*) cell dry weight.

the maximum ethanol concentrations achieved were 7.14 and 7.10 g/L, respectively, with a similar yield of 0.42 g ethanol/g glucose. However, at the medium inoculum size (15 and 20%), yeast utilized all the hydrolysate glucose after 9 h of fermentation, and produced the higher ethanol titers, 8.47 and 8.62 g/L with a maximum yield of 0.45 and 0.46 g ethanol/g glucose, respectively. Not only the final ethanol yield and titer were high, but the productivity values (0.94 and 0.95 g/L/h^{-1}) obtained for 15 and 20% inocula were also higher than for all other inoculums sizes (Table 2, Fig. 1b). For the small size inocula (1 and 7%),

Table 2. Effect of the *S. cerevisiae* 2.399 inoculum size on maximum ethanol titer, yield, and productivity

Inoculum size, % (vol/vol)	Initial glucose concentration, g/L	Time to get maximum ethanol, h	Maximum ethanol titer, g/L	Yield, g ethanol/g glucose	Productivity, g/L/h^{-1}
1	17.65 ± 1.33	21	7.47 ± 0.33	0.42	0.35
7	17.70 ± 0.89	12	7.75 ± 0.30	0.43	0.64
15	18.80 ± 1.03	9	8.47 ± 0.38	0.45	0.94
20	18.62 ± 0.90	9	8.62 ± 0.33	0.46	0.95
25	17.01 ± 1.15	6	7.14 ± 0.39	0.42	1.19
30	16.90 ± 1.07	6	7.10 ± 0.47	0.42	1.18

Table 3. Effect of pH of the *S. cerevisiae* 2.399 growth medium on maximum ethanol titer, yield, and productivity

pH	Initial glucose concentration, g/L	Time to get maximum ethanol, h	Maximum ethanol titer, g/L	Yield, g ethanol/g glucose	Productivity, g/L/h^{-1}
4.0	17.50 ± 1.19	18	7.0 ± 0.42	0.40	0.38
5.0	18.0 ± 1.04	9	7.93 ± 0.50	0.44	0.88
6.0	18.60 ± 0.95	9	8.57 ± 0.66	0.46	0.95
7.0	17.30 ± 1.10	9	7.46 ± 0.48	0.43	0.82
8.0	17.20 ± 1.38	9	7.32 ± 0.43	0.42	0.81

the fermentation was slow and took more time to complete. The final ethanol concentration and yield for 1% inoculum were 7.47 g/L and 0.42 g ethanol/g glucose after 21 h of fermentation (Table 2), while 7% inoculum produced 7.75 g/L ethanol with a final yield of 0.43 g ethanol/g glucose after 12 h (Fig. 1c, Table 2).

From the above results, we can conclude that there is a direct relationship among the inoculum concentration, yeast biomass, and final yield and concentration of ethanol, provided that the optimum limit of inoculum concentration is not breached. The more obvious effect of the increase in inoculum concentration was the faster fermentation process, more yeast cell biomass, along with some increase in the yield and titer of ethanol. This might be attributed to the fact that the higher initial inoculum concentration (not exceeding the optimum level) results in a rapid multiplication of the yeast cells due to a rapid consumption of the nutrients. The same observation was also reported by Arshad et al. [29] and Laluce et al. [30] using *S. cerevisiae* to ferment blackstrap molasses and glucose/sucrose, respectively. Laluce et al. [30] explained that the increase in ethanol might not only be due to the positive relationship between high inoculum size and glucose, but also, on the other hand, the severity of ethanol inhibition is reduced in high-cell density cultures, leading to an overall increase in viability of microbial cells. On the basis of this observation, we can infer that interaction between hydrolysate glucose and yeast cells was very positive in 15 and 20% inocula. Based on its high yield and final ethanol titer, 20% inoculum was considered as the optimum inoculum level for the fermentation of hydrolysate glucose.

Effect of pH on hydrolysate fermentation. Improved ethanol fermentation can be achieved by controlling various parameters; among them, pH is also one of the important parameters [31]. Hydrolysate fermentation was carried out at various pH levels of 4.0, 5.0, 6.0, 7.0 and 8.0 using the previously optimized 20% vol/vol inoculum concentration. Figure 2 shows the effect of various pH levels on fermentation of hydrolysate glucose after 6, 9 and 18 h of fermentation. After 3 h of fermentation, the tendency of growth and hydrolysate fermentation was very positive in all pH media except pH 4.0. At 6 h of fermentation, a rapid decrease in hydrolysate glucose concentration was observed for pH from 5.0 to 8.0, and some ethanol was also produced. However, very little ethanol was produced at pH 4.0, indicating that the growth and ethanol fermentation were extremely slow (Fig. 2a). With the passage of time, the growth, and ethanol titer started to improve for all the pH levels above 4.0. Table 3 shows the values of yield, productivity and final ethanol concentration at various pH values. At 9 h of fermentation, no glucose was detected in media with pH from 5.0 to 8.0, although the hydrolysate glucose was consumed at the same time, however the final concentration and yield of ethanol was not the same for different pHs. Maximum ethanol concentration (8.57 g/L) was achieved for medium with pH 6.0 followed by 7.93 g/L at pH 5.0 (Fig. 2b, Table 3). As stated earlier, the fermentation was very slow at pH 4.0, and it took

about 18 h to produce the final ethanol titer i.e. 7.0 g/L (Fig. 2c, Table 3).

In our pH experiment, we observed that the incubation time required for growth and the maximum ethanol concentration was prolonged at pH 4.0; however, the final concentration of ethanol was not too small at the low pH level. The same observation was also reported by Lin et al. [32] using *S. cerevisiae* to ferment glucose. Previous research has reported that the optimal pH range for the growth of yeast can vary from 4.0 to 6.0, depending on the strain of the yeast, availability of oxygen, and the temperature [33]. Based on its high yield and productivity, pH 6.0 was the optimum level for the fermentation of the hydrolysate glucose. Table 4 shows that most of the previous studies regarding bio-oil hydrolysate fermentation using yeast have been carried out at pH 5.5 or 6.0, which further validates the results of this study.

Effect of hydrolysate glucose concentration on ethanol fermentation. In addition to the starting inoculum concentration, initial substrate concentration is also a key factor that greatly influences the yield and productivity of fermentation products. However, research in the recent past has considered efficient substrate utilization as a major factor for optimum biofuel production [34]. It is an economically relevant factor to know the maximum concentration of hydrolysate glucose that could be efficiently fermented by the yeast. For this purpose, fermentation was carried out at different concentrations of hydrolysate glucose (2, 4, 6, 8, and 10%) in shake flasks at pH 6.0 and 20% (vol/vol) inoculum concentration. In our experiments, we observed that concentration of hydrolysate glucose always decreased after media autoclaving, which might be attributed to Maillard reaction. Therefore, in order to make our experimental values more precise, we detected the initial hydrolysate glucose concentration at 0 h after inoculation with yeast. Figure 3 shows the effect of different concentrations of hydrolysate glucose on ethanol and cell biomass production after 8 and 16 h of fermentation. The fermentation for 2% hydrolysate glucose was very rapid; all the glucose was completely consumed after 4 h (data not shown) of fermentation, while maximum ethanol concentration (7.83 g/L) was detected at 8 h (Fig. 3a). On the other hand, 4% hydrolysate glucose was completely utilized after 12 h of fermentation (data not shown), producing a final ethanol titer of 14.78 g/L at 16 h (Fig. 3b). The effect of hydrolysate glucose on maximum ethanol titer, yield, and productivity are given in Table 5. For 6 and 8% hydrolysate glucose, a drop in the initial glucose concentration was observed at the start of fermentation, but later on there was no change in the concentration of glucose, even after 5 days of fermentation. The cell biomass and titer of ethanol were both very low, just 3.45 g/L ethanol was produced for 6% hydrolysate glucose after 56 h of fermentation, while 2.84 g/L ethanol was produced in the media containing 8% hydrolysate glucose after 72 h of fermentation

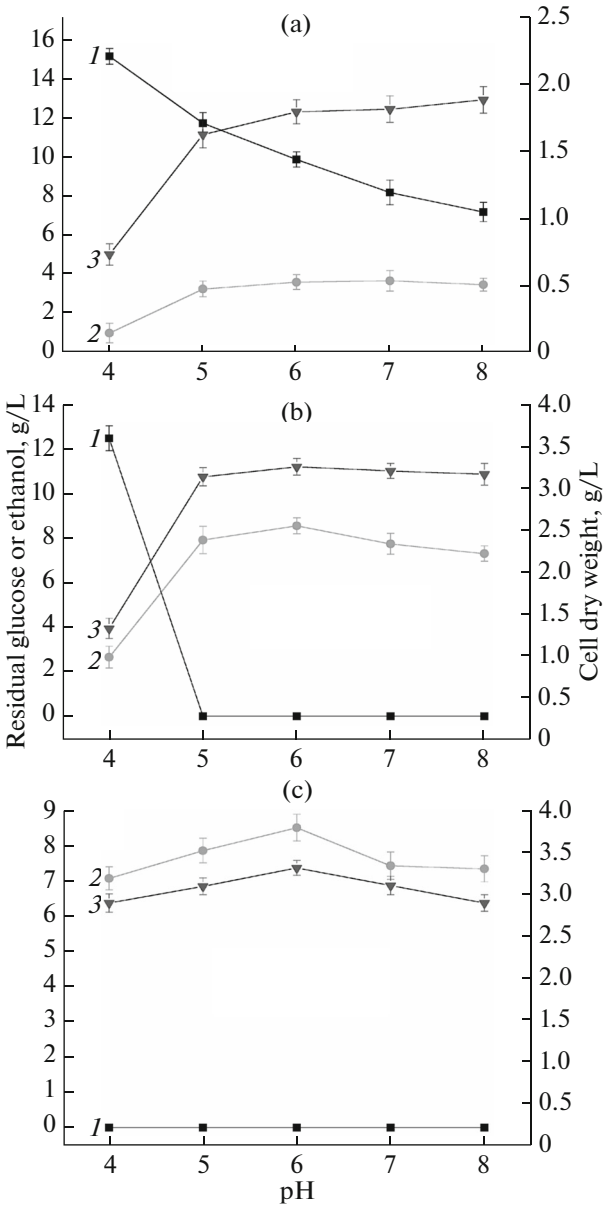

Fig. 2. Effect of pH of the *S. cerevisiae* 2.399 growth medium on ethanol fermentation of detoxified hydrolysate glucose after 6 (a), 9 (b) and 18 h (c). (*1*) Residual glucose; (*2*) ethanol; (*3*) cell dry weight.

(Table 5). The similar picture was observed with 10% hydrolysate glucose; however no ethanol was detected at any stage of fermentation. With respect to the titer and yield of ethanol, 4% hydrolysate glucose concentration could be regarded as the optimum level that could be efficiently fermented by the yeast.

Table 4 shows the maximum concentration of hydrolysate glucose that has been efficiently fermented to ethanol in the previous studies in comparison to our results. Most of the previous studies have reported the fermentation of 2, 3, or 4% hydrolysate glucose. Only one study by Lian et al. [17] has reported

Table 4. Comparison of results of hydrolysate fermentation performed in this study with the previous research*

Inoculum	pH	Hydrolysate glucose, g/L	Microorganism	Type of fermentation	Time for max. ethanol titer, h	Max. ethanol titer, g/L	Ethanol yield, g ethanol/g glucose	Productivity, $g/L/h^{-1}$	Reference
10%	6.0	35.9	*S. cerevisiae* 2.399	Flask	24	16.1	0.45	0.67	[21]
10%	6.0	31.6	*S. cerevisiae* 2.399	Flask	24	14.2	0.45	0.59	[22]
1 g/L	5.5	20%	*S. cerevisiae* T2	Flask	NM	NM	0.46	0.55	[23]
2.5 g/L	5.5	40%	*S. cerevisiae* T2	Flask	NM	NM	0.45	NM	[24]
10%	7.0	70	*S. cerevisiae* ATCC 200062	Flask	20	32–35	0.47	1.6	[25]
10%	NM	40	*S. cerevisiae* DSM 1334	Microtiter plate wells	15	20	0.49	1.33	[29]
$0.5OD_{600}$	6.7	20	*Saccharomyces pastorianus* ATCC 2345	Flask	36	12.12	0.5	0.34	[28]
10%	7.0	37.13	*Escherihia coli* ACCC 11177	Fermenter	18	14.97	0.41	0.83	[5]
20%	6.0	32.01/29.29	*S. cerevisiae* 2.399	Flask/Fermenter	16/10	14.78/13.23	0.46/0.45	0.92/1.32	This study

* NM—not mentioned.

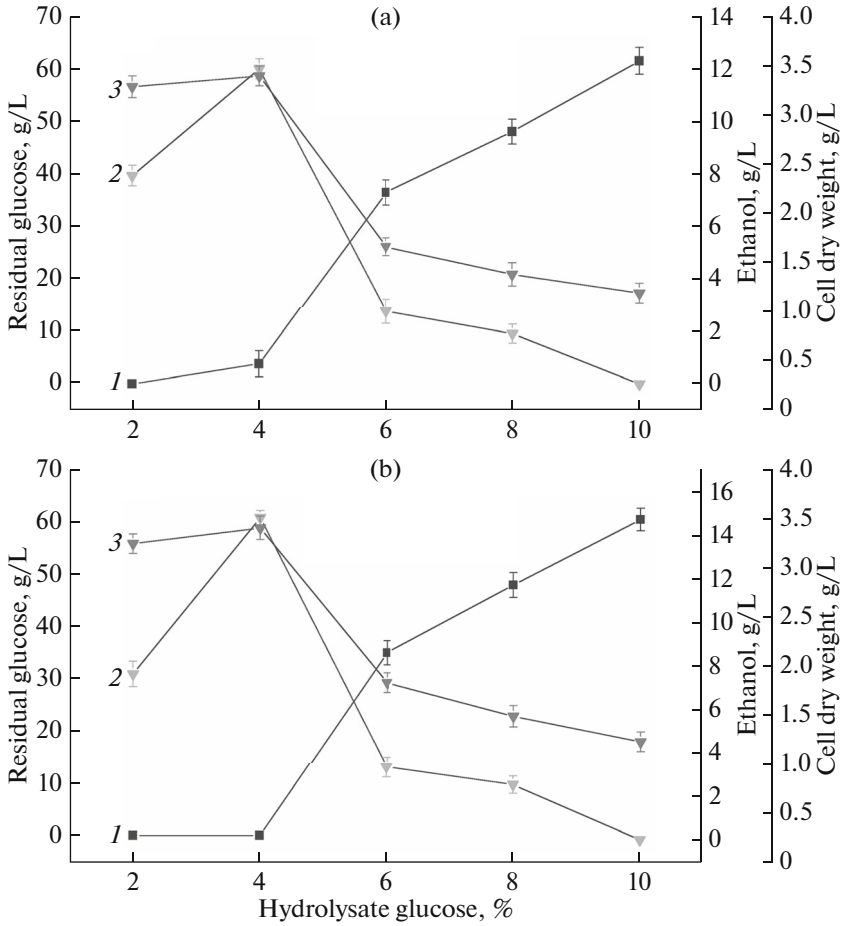

Fig. 3. Effect of different concentrations of hydrolysate glucose on ethanol fermentation after 8 (a) and 16 h of fermentation (b).

the successful fermentation of a higher concentration of bio-oil hydrolysate glucose (7%) with a higher yield and productivity, but it might be attributed to the type of bio-oil, its chemical composition and the strain of the yeast. From the above results, we can conclude that increasing the concentration of hydrolysate glucose (above 4%) results in a decrease in the bioconversion efficiency, which might be attributed to the complex chemical composition of the pyrolysate. Increasing the concentration of hydrolysate glucose means an increase in the chemical complexity of the fermentation medium, which would in turn cause an increase in osmotic pressure on the yeast cell wall. The increased osmotic pressure may cause the inhibition of ethanol to diffuse out from the yeast cells, hence leading to a reduced growth and an overall decline in the ethanol fermentation due to intracellular accumulation of ethanol [35]. This could be the probable explanation for

Table 5. Effect of hydrolysate glucose used in the *S. cerevisiae* 2.399 growth medium on maximum ethanol titer, yield, and productivity*

Glucose, %	Initial glucose concentration, g/L	Time to get maximum ethanol, h	Maximum ethanol titer, g/L	Yield, g ethanol/g glucose	Productivity, $g/L/h^{-1}$
2.0	17.20 ± 0.88	8	7.83 ± 0.39	0.46	0.97
4.0	32.01 ± 1.10	16	14.78 ± 0.33	0.46	0.92
6.0	47.54 ± 1.81	56	3.45 ± 0.45	0.24	0.06
8.0	60.41 ± 1.56	72	2.84 ± 0.51	0.21	0.03
10.0	72.68 ± 1.73	Nil	Nil	Nil	Nil

* Nil—No ethanol detected.

Table 6. Fermentation of pure glucose and hydrolysate glucose in fermenter using *S. cerevisiae* 2.399

Fermentation time, h	Pure glucose as control			Hydrolysate glucose		
	residual glucose, g/L	cell dry weight, g/L	ethanol, g/L	residual glucose, g/L	cell dry weight, g/L	ethanol, g/L
0	32.36 ± 1.43	0.60 ± 0.11	0	29.29 ± 1.32	0.73 ± 0.13	0
2	22.17 ± 1.10	1.30 ± 0.10	2.62 ± 0.69	24.02 ± 1.19	1.06 ± 0.10	1.98 ± 0.59
4	13.52 ± 1.19	2.19 ± 0.15	5.70 ± 0.33	18.05 ± 1.27	1.89 ± 0.19	3.33 ± 0.68
6	3.71 ± 1.28	3.33 ± 0.20	10.63 ± 0.40	12.65 ± 1.23	2.81 ± 0.21	5.85 ± 0.39
8	1.10 ± 1.33	4.20 ± 0.26	12.89 ± 0.38	4.45 ± 1.36	3.63 ± 0.12	9.98 ± 0.24
10	0	4.18 ± 0.17	15.32 ± 0.25	0	3.59 ± 0.20	13.23 ± 0.51
12	0	4.19 ± 0.10	15.30 ± 0.55	0	3.61 ± 0.31	13.20 ± 0.38
14	0	4.15 ± 0.21	15.30 ± 0.41	0	3.58 ± 0.15	13.17 ± 0.63

Table 7. Model parameters for pure glucose fermentation by *S. cerevisiae* 2.399

Biomass		Residual glucose		Ethanol	
name	value	name	value	name	value
X_0	0.626	k^{st}	0.513	k^{st}	−3.26E-8
μ_{max}	0.324	k^{div}	−2.44	k^{div}	1.06
t_{Lim}	5.01	P_{Lim}	8.95	P_{Lim}	8.04
X_{Lim}	3.17	P_{Fin}	0.485	P_{Fin}	14.6
X^{st}_{Lim}	1.41				
A	0.22				
X_P	4.75				
t_{Fin}	10				
R_{Fin}	1				

the extremely low concentration or without ethanol titer at higher concentrations of hydrolysate glucose (6, 8 and 10%).

Fermentation of Hydrolysate in Fermenter

Batch fermentation was carried out in 7-L capacity stirred fermenter with 3-L working volume. Table 6 shows the fermentation of pure glucose as control and the hydrolysate glucose in fermenter. In both cases, the glucose was completely fermented at the same time i.e. 10 h. The concentration and yield of ethanol in pure glucose medium were 15.32 g/L, and 0.47 g ethanol/ g glucose, respectively. On the other hand, the titer and yield of ethanol for the hydrolysate medium in the fermenter were lower compared to control. The yield of ethanol reported in shake flask fermentation for hydrolysate glucose was 0.46 g ethanol/g glucose, but in the fermenter it decreased to 0.45 g ethanol/g glucose, suggesting that the bioconversion efficiency of hydrolysate glucose to ethanol decreased as the scale of fermentation was increased. However, the process of fermentation was more efficient and completed in relatively short time compared to the process in shake flasks, which might be attributed to the well-controlled fermentation conditions in fermenter. The productivity for the fermentation of hydrolysate glucose in the fermenter was 1.32 g/L/h^{-1}, which was higher than the shake flasks (0.92 g/L/h^{-1}). Only Lian et al [17] and Luque et al [21] could report higher productivity values than this study, however, they carried out hydrolysate fermentation on a small scale (Table 4).

The values of model parameters for the pure glucose and hydrolysate glucose are listed in Tables 7 and 8, respectively. According to the mathematical model parameters, the maximum specific growth rate μ_{max} is 0.324 and 0.222 h^{-1} for pure glucose and hydrolysate medium, respectively, indicating that the pure glucose medium is more favorable for the growth of the yeast. On the other hand, the rate of ethanol synthesis con-

Table 8. Model parameters for hydrolysate glucose fermentation by *S. cerevisiae* 2.399

Biomass		Residual glucose		Ethanol	
name	value	name	value	name	value
X_0	0.68	k^{st}	6.95E-09	k^{st}	−7.09E-9
μ_{max}	0.222	k^{div}	−2.82	k^{div}	1.14
t_{Lim}	3.85	P_{Lim}	20.4	P_{Lim}	3.28
X_{Lim}	1.6	P_{Fin}	4.41	P_{Fin}	9.71
X^{st}_{Lim}	0.736				
A	0.289				
X_P	4.44				
t_{Fin}	8				
R_{Fin}	0.681				

stant k^{div} is 1.14 h^{-1}, which is slightly higher than the corresponding k^{div} for the control medium (1.06 h^{-1}) indicating that hydrolysate glucose was efficiently fermented into ethanol. The constant of product degradation k^{st} is almost equal to 0, implying that there was no ethanol degradation for both types of media. The constant for glucose utilization (k^{div} for glucose) is −2.44 h^{-1}, that is smaller than the for the hydrolysate glucose (−2.82 h^{-1}), which indicates that the specific glucose consumption rate for the pure glucose fermentation is higher than for the hydrolysate glucose. The parameters discussed above are a little more favorable to reach the greater efficiency of the ethanol synthesis in the hydrolysate medium rather than in the pure glucose.

It should be noted that in Table 8 we see maximum value $P_{Fin} = 9.71$, which corresponds to the maximum value of the concentration of ethanol at the end of GIP phase. However, in the late of stationary phase concentration of ethanol corresponds to ~15 g/L, as in the case of glucose. This suggests that the process in the hydrolysate medium is not worse, than the process in glucose. Statistical analyses are an essential tool for assessing data for mathematical models. The correlation coefficient R^2, for the ethanol fermentation from pure glucose is equal 0.998. The correlation coefficient R^2, for ethanol from hydrolysate glucose is equal 0.993. The confidence of probability for both processes is 95%. Similar calculations were performed earlier using criterion for the Fisher method and have shown adequacy of the model [23].

In conclusion, the hydrolysate glucose was successfully fermented in the shake flasks as well the fermenter after detoxification with ethyl acetate and activated carbon. The optimum inoculum concentration was found to be 20% vol/vol, and pH 6.0 was the best for optimal ethanol fermentation. Hydrolysate glucose concentration up to 4% was the optimum level that could be successfully fermented into ethanol by *S. cerevisiae* 2.399. Although most of the already known inhibitors were almost completely removed after ethyl acetate treatment, even then, the yeast could not ferment hydrolysate glucose above 4% concentration, suggesting that the chemical complexity of pyrolysate must be reduced. Scale-up studies in stirred fermenter produced much higher productivity (1.32 g/L/h^{-1}) compared to shake flask fermentation (0.92 g/L/h^{-1}).

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