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Fermentation of Xylose and Glucose Mixture in Intensified Reactors by *Scheffersomyces stipitis* to Produce Ethanol

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***Abstract*—**In this work, two fermentations at different temperatures (25 and 30ºC), with cell recycling, were accomplished to produce ethanol, using a mix of commercial substrates, xylose (70%) and glucose (30%), as organic source for *Scheffersomyces stipitis*. Five consecutive fermentations of 80 g L^{-1} (1º, 2º and 3º recycles), 96 g L^{-1} (4º recycle) and 120 g L^{-1} (5º recycle)reduced sugars led to a final maximum ethanol concentration of 17.2 and 34.5 g L^{-1}, at 25 and 30ºC, respectively. Glucose was the preferred substrate; moreover xylose startup degradation was initiated after a remaining glucose presence in the medium. Results showed that yeast acid treatment, performed before each cycle, provided improvements on cell viability, accompanied by ethanol productivity of 2.16 g L^{-1} h^{-1} at 30ºC. A maximum 36% of xylose was retained in the fermentation medium and after five-cycle fermentation an ethanol yield of 0.43 g ethanol/g sugars was observed. *S. stipitis* fermentation capacity and tolerance showed better results at 30ºC with 83.4% of theoretical yield referenced on initial biomass.

***Keywords*—**5-carbon sugar, cell recycling fermenter, mixed sugars, xylose-fermenting yeast.

I. Introduction

DUE to increasing population added to the industrialization need, energy demand is increasing frequently and concomitantly is the raise of the worldwide second generation ethanol production efforts [1]. Lignocellulosic biomass is an abundant, renewable feedstock with a potential source of carbohydrates (cellulose and hemicellulose), including glucose and xylose [2], for the production of biofuels and value-added chemicals, if an efficient and approachable conversion technology can be established to overcome its recalcitrance [3].

Whereas ethanolic fermentation of hexoses derived from cellulosic biomass, i.e., glucose, mannose and galactose, using the yeast *Saccharomyces cerevisiae* is well established on large scale, pentose conversion (e.g., xylose and arabinose) to ethanol and its complete utilization is still one of the major obstacles to industrializing the lignocellulosic ethanol production because of hydrolysates complexity [4], [5]. Furthermore, an efficient microorganism, capable of degrading biomass efficiently and in high yields, is necessary to facilitate a sustainable and economically viable manufacturing of biofuels and bioproducts from lignocellulosic materials [6].

Scheffersomyces stipitis yeast has been widely studied for its pentose fermenting capacity. Some of the available studies used xylose as exclusive carbon source [5] or as a combination with glucose [7], [8]. In yeasts, transport systems for hexoses also conduct pentoses, and beyond that it is a well-known insight that xylose uptake can only befall after hexoses have been significantly diminished from the medium [9]. Recently, *S. stipitis* strains have shown to be a potential 2G ethanol producing microorganism from a range of lignocellulosic hydrolysates including hardwood spent sulfite liquor [10], [11], sugarcane bagasse [12]-[14], corncob [15], giant reed [16], rice straw [17] and rice husk [18].

Nevertheless, inhibitor tolerance is a critical framework when fermenting sugars from pretreated lignocellulosic material using yeasts [19], as *Scheffersomyces stipitis*. This specie has a low tolerance to ethanol and sugar, which has restricted its use as an industrial strain for large-scale bioethanol production from xylose [5]. Additionally, the use of real substrates for 2G ethanol production may form, during the lignocellulosic biomass pretreatment, inhibitor compounds (acetic acid, furfural, 5-hydroxymethylfurfural and phenolic compounds) [20]-[22], which it is associated to microbial growth rate reduction of *S. stipitis* and thus, the fermentation viability decrease [23].

An operational strategy that could reduce inhibitor intrinsic effects on xylose fermentation by *S. stipitis* is cell recycle fermentation. The recycle of cells, richly used in 1G ethanol production [24], enables microorganism adaptation and conditioning to a possible deleterious intrinsic status of the fermentation system [8]. This process also reduces significantly the fermentation time and costs associated with the inoculum preparation [25]-[27]. Although it is a technique with widespread strains of *Saccharomyces cerevisiae*, few studies have been conducted with regard to the fermentation through a cell recycle batch system using *S. stipitis*. Another factor is related to the applied temperature. Temperatures lower than those prevailing industrial operation (30°C) could improve the ethanol tolerance of *S. stipitis.*

Therefore, the aim of this study was to evaluate the capacity

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of the wild *Scheffersomyces stipitis* NRRL Y7124 strain to produce ethanol during five cell recycle fermentations using mixed sugars as organic source, xylose and glucose, at two temperatures, 25 and 30ºC, as an attempted to improve ethanol productivity, yield and yeast cell viability.

II. Material and Methods

A. Strain and Inoculum

The yeast used in this study was the wild strain *Scheffersomyces stipitis* NRRL Y7124, a xylose-fermenting microorganism.

Yeast cultures were maintained on solid YPX agar medium at 4ºC. For pre-inoculum preparation to activate the yeast, cells from the solid medium were transferred to 250 mL Erlenmeyer flasks containing 100 mL of YPX medium, incubated during 24 h, at 30ºC and 200 rpm. After this period, 10 % (v/v) of the pre-inoculum was transferred to the inoculum medium (adapted from [28]) composed by (g L^{-1}): xylose (12.0), glucose (1.32), urea (2.3), yeast extract (3.0) and $MgSO_4.7H_2O$ (1.0). The inoculated flasks were kept at 30ºC and 200 rpm, for a period of 24 h.

The following step included the centrifugation of the entire inoculum volume at 8000 rpm, 4ºC for 20 min. Next, the cells were re-suspended in sterile water and centrifuged under the same conditions. These cells were re-suspended in sterile water (10% of the reactor working fermentation volume) and were direct to a prior system at 25 and 30°C alcoholic fermentation: a biocatalyst system for high microbial mass of *S. stipitis*.

B. Preliminary Step: Growth Conditions

At this stage, before fermentation starts, a biocatalyst system for high microbial mass of *S. stipitis* was established. The dissolved oxygen percentage was maintained at 40% to avoid oxygen limitation. The growth medium composition was composed by (g L^{-1}): sugarcane syrup (30.0), KCl (1.0), $CaCl_4.2H_2O$ (1.0), trace elements (1.0 mL L^{-1}), thiamine (0.003), a pulse of KH_2PO_4 (2.0) and urea (5.0).

A continuous feeding (3 g of reducing sugars – RS - L^{-1} h^{-1}) was activated in order to maintain the limiting carbon and control linear growth. Thus, after this procedure to establish a high cell mass *S. stipitis*, the cell concentration in the reactor to start the fermentations at 25 and 30°C, were 10.1 and 13.08 g L^{-1}, respectively.

C. Fermentation

To investigate the ethanol production by *S. stipitis*, two fermentations were carried out under different temperatures (25 and 30ºC) using five consecutive cells recycles fermentations. Assays were performed in reactors BioFlo® 115 (New Brunswick Scientific Co., Inc., Edison, NJ) with 1.4 L of working volume comprising the inoculum obtained from the biocatalyst system for high microbial mass added to the fermentation medium according to Table I (adapted from [28]). Thus, the reducing sugars concentrations was 80 g L^{-1} during the first three recycles, and then, for the fourth and fifth recycle, the concentrations were 96 and 120 g L^{-1}, respectively. During fermentations, the conditions of agitation and aeration were 200 rpm and 0.1 vvm, respectively, with a volumetric oxygen transfer coefficient (K_La) of 4.9.

TABLE I
Fermentation Mediums Composition and Concentrations along Ethanol Production from Xylose and Glucose by *S. stipitis* under Cell Recycling

Compounds	Concentration (g L^{-1})		
	1º, 2º and 3º recycles	4º recycle	5º recycle
Xylose	56	67	83
Glucose	24	29	37
Yeast extract	3.0	3.0	3.0
$MgSO_4.7H_2O$	1.0	1.0	1.0
Urea	2.3	2.3	2.3

After the fermentation run monitored by the exhaustion of glucose and consumption of xylose as carbon sources, and also by the stability of the ethanol production, the agitation was turned off and the fermentative solution was pumped out from the bioreactor to a sealed and sterile flask through a peristaltic pump. The broth was centrifuged at 7500 rpm for 20 min. The precipitate cells were removed, re-suspended with sterile water and subjected to an acid treatment, followed by fermentation for the upcoming recycle. This procedure was repeated 5 times (1st, 2nd, 3rd, 4th and 5th recycle).

D. Acid Treatment

Yeast cells were treated with sulfuric acid (2 M), which was intended to eliminate flocculation and possible stresses caused to the yeast during the fermentative process. The acid treatment was accomplished before the beginning of each of the fermentations, with the following conditions: 30 °C, 200 rpm, 0.14 vvm and pH 2.5 maintained during 30 min. After this period, the cells were removed from the bioreactor via peristaltic pump to a sterile flask and centrifuged at 7500 rpm for 20 min. The cells obtained were re-suspended in sterile water and again subjected to centrifugation at 7500 rpm for 20 min. The precipitate biomass of *S. stipitis* was suspended in sterile water, pumped back to the bioreactor to be used as inoculum to the subsequent batch fermentation.

E. Analytical Methodology

Throughout the fermentations with *S. stipitis* at different temperatures (25 and 30ºC), periodic physical and chemical analysis were performed on samples collected from the rectors. Determination of parameters involving yeasts and metabolites concentrations, pH, agitation, dissolved oxygen, temperature and microaerophilic measurements were performed at the starting point (0 h fermentation) and every 4 h elapsed from the fermentation start until the assay finalization. Furthermore, for the purpose of monitoring organic substrates consumption, glucose and xylose concentrations were verified by middle infrared (FT-IR Spectrometer – Alpha, OPUS software, Bruker Optics Co.) every 2 h until glucose exhaustion. After this finding, samples were taken each 4 h.

Yeasts concentrations were measured by gravimetrically analysis in triplicate, whereas 2 mL of each sample were

submitted to centrifugation (4000 rpm for 3.5 min), re-suspended twice in distilled water and directed to biomass drying oven at 80°C.

For greater accuracy and specificity of carbohydrates, organic acids and alcohols concentrations obtained during fermentation process, analysis of glucose, xylose, acetic acid, glycerol, xylitol and ethanol were determined by high performance liquid chromatography (Agilent Infinity 1260, Santa Clara, CA, USA). Samples were filtered through a Millex 22um PVDF filter, and the filtrate was injected into the HPLC system. Compounds were separated with an Aminex HPX 87H (300 7.8 mm, BIO-RAD, Hercules, CA) at 35 ºC using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1}.

F. Cell Viability

The *Scheffersomyces stipitis* viability was checked by optical microscopy views in 40X objective, considering the viable and non-viable cells, viable and non-viable budding cell. Counting was effected by means of a Neubauer chamber and methylene blue dye was used to differentiate living from dead cells.

III. Result and Discussion

A. Fermentation at 25ºC

An overview of the values obtained in the five recycles fermentation at 30°C regarding ethanol production by *S. stipitis* is presented in Table II.

Total time duration of the fermentation assay at 25 °C was 137 h. Ethanol productivity values obtained were between 0.19 and 1.01 g L^{-1} h^{-1} and EtOH yield was found between 61 and 81%. As can be seen in Fig. 1, significant increase on ethanol production and substrate consumption (reducing sugars – RS) were achieved along the cell recycles with *S. stipitis*, with a maximum ethanol production of 17.82 g L^{-1} obtained at the fourth recycle.

TABLE II
Fermentation Parameters Achieved during Ethanol Production at 25ºC from Xylose and Glucose by *S. stipitis* under Cell Recycling

Recycle	Fermentation time (h)	Maximum EtOH concentration (g L^{-1})	Q_P [a] (g L^{-1} h^{-1})	$Y_{(P/S)}$ [b] (g g^{-1})
1	19.32	3.60	0.19	0.42
2	24.00	13.15	0.52	0.33
3	23.58	6.33	0.21	0.38
4	24.27	17.82	1.01	0.31
5	46.76	17.20	0.35	0.39

a QP: ethanol volumetric productivity
b Y(P/S): ethanol yield factor

Silva et al. [28] evaluated the fermentation medium and oxygen transfer conditions that maximize xylose conversion to ethanol by *S. stipitis*. The authors reported an ethanol yield of 0.32 g g^{-1} with a volumetric oxygen transfer coefficient (K_La) of 4.9. In the present work under the studied conditions, it was possible to observe higher values of 0.42, 0.33, 0.38, 0.31 and 0.39 g g^{-1}as ethanol yield factors during the 1º, 2º, 3º, 4º and 5º cells recycle, respectively. According to previous studies, when high concentrations of cells are applied in recycling systems, the strains have a greater survival chance against toxic environment formed by inhibitory compounds [30]. Additionally, the recycling system has been shown to favor strains cell growth of microorganisms with desirable performance degradation [25].

At the fourth recycle batch, it was possible to achieve the maximum values for ethanol production and productivity (17.82 g L^{-1} and 1.01 g L^{-1} h^{-1}, respectively). Thus, glucose and xylose consumption, ethanol, dry weight cell mass and xylitol concentrations during fermentation at the 4º recycle can be notice at Fig. 2. An important regulatory role in the metabolism of *S. stipitis* was performed by glucose concentration in the medium and its consumption. Glucose was promptly metabolized and probably inhibited the expression of related genes encoding enzymes of the pentose metabolism. However, after low glucose concentration condition in the medium, probably enzymes de-repression in the biosynthesis occurred, enabling a wide variety of regulatory phenomena [29], favoring the 2G ethanol production from pentose.

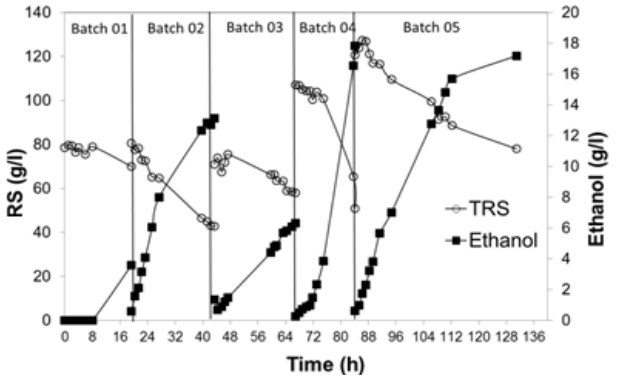

Fig. 1 Ethanol production and reducing sugars consumption (g L^{-1}) during fermentation at 25ºC by *Scheffersomyces stipitis* using glucose and xylose as substrates

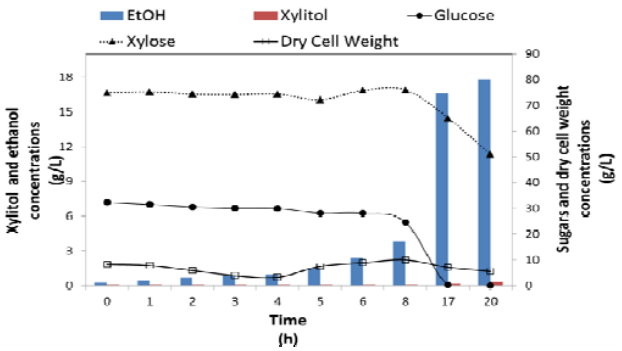

Fig. 2 Glucose and xylose consumption, ethanol, dry weight cell mass and xylitol concentrations during fermentation at the 4º recycle batch at 25ºC

Agbogbo et al. [7] also observed this behavior in xylose and glucose mixing fermentation with similar percentages from the ones used in the present study, 75 and 25%, respectively,

accompanied by ethanol production of 24.4 g L^{-1}. This trend caused by glucose catabolic repression was detected in all five recycles, as well as low xylitol concentrations produced (from 0.0 to 0.5 g L^{-1}), acetic acid (0.0 to 0.6 g L^{-1}) and stability of *S. stipitis* cell dry weight mass (minimum of 5.57 g L^{-1} and maximum of 13.65 g L^{-1}).

Cell viability remained constantly elevated between 76 and 92%, with budding rate percentages between 1.5 and 18.0%, a condition that can be attributed to the acid treatment performed before each recycle start. Fig. 3 (a) shows yeast cells flocculation at the end of the fermentation process.

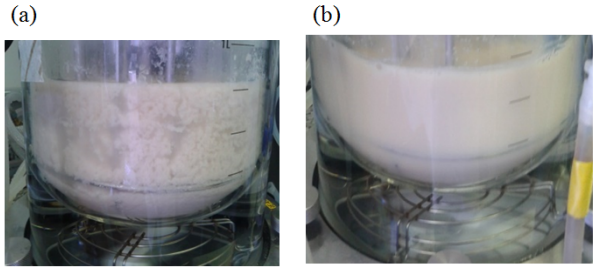

Fig. 3 (a) Cells flocculation at fermentation process end and (b) flocculation disruption after acid treatment

After acid treatment, flocculation disruption was observed and possible inhibitory compounds removal from intracellular medium might occur (Fig. 3 (b)). Optical microscopic observation displayed that *S. stipitis* cells remained viable even after the fifth recycling.

B. Fermentation at 30ºC

The uptake of reducing sugars (RS) and the consequent ethanol production can be checked by observing Fig. 4. Over the successive recycles is noticeable and clear the behavior decline in the concentration of reducing sugars with consequent ethanol production. Xylose percentages consumed during the 1º, 2º, 3º, 4º and 5º batch fermentations were 15.76%, 39.87%, 63.20%, 60.91% and 49.11%, respectively. Despite the similar trend compared to the fermentation at 25ºC, and also with the glucose catabolic repression, the process at 30°C showed higher values of ethanol production, productivity and yield.

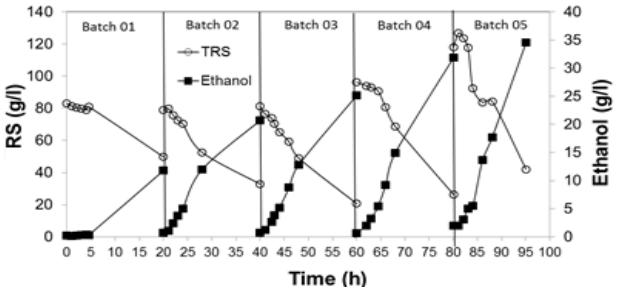

Fig. 4 Ethanol production and reduced sugars consumption (g L^{-1}) during fermentation at 30 ºC by *Scheffersomyces stipitis* using glucose and xylose as substrates

As observed at Table III, the maximum ethanol concentration achieved was 34.54 g L^{-1}. The total time duration of fermentation was 95 hours. Ethanol yield factor attained values of 0.35 to 0.45 g g^{-1}. The results obtained from the fermentation at 30 ºC were higher to the ones achieved at 25 ºC. Even so, the data represents a perspective to improve ethanol production from xylose. In this sense, [31] affirmed that improvements of ethanol-fermentation microorganisms and bioreactors design and techniques should be co-developed to cause a significant effect on ethanol production viability.

TABLE III
FERMENTATION PARAMETERS ACHIEVED DURING ETHANOL PRODUCTION AT 30ºC FROM XYLOSE AND GLUCOSE BY *S. STIPITIS* UNDER CELL RECYCLING

Recycle	Fermentation time (h)	Maximum EtOH concentration (g L^{-1})	Q_P [a] (g L^{-1} h^{-1})	$Y_{(P/S)}$ [b] (g g^{-1})
1	20.00	11.78	0.57	0.35
2	20.00	20.69	0.99	0.43
3	20.00	25.13	1.21	0.40
4	20.07	31.87	1.78	0.45
5	15.00	34.54	2.16	0.43

a QP: ethanol volumetric productivity
b Y(P/S): ethanol yield factor

Fig. 5 presents the ethanol productivity and yields progressive increases along the successive fermentations cells recycles at 30ºC. EtOH yield and productivity were found from 68 to 87% and between 0.57 and 2.16 g L^{-1} h^{-1}, respectively.

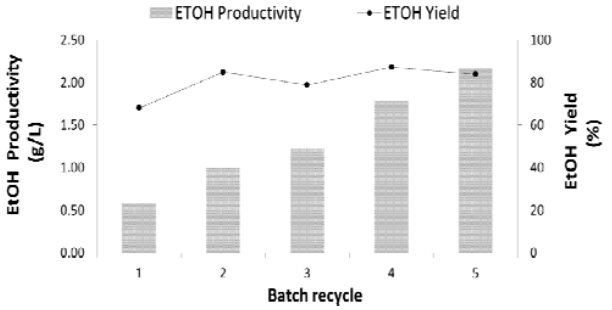

Fig. 5 Ethanol productivity (g L^{-1}) and yield (%) during fermentation at 30ºC by *Scheffersomyces stipitis* using glucose and xylose as substrates

Xylitol and acetic acid concentrations remained at minimal concentrations, from 0.0 to 2.4 g L^{-1} and between 0.0 to 0.35 g L^{-1}, respectively. *Scheffersomyces stipitis* cell viability remained constant and adequate, as occurred at 25ºC fermentation, with percentages between 65 and 93%, with budding rate percentages between 2.5 and 19.6% (Fig. 6). It is possible to observe that the acid treatment strongly and positively influenced the cell perform and viability. At all batches beginnings, it was revealed increased cell viability after acid treatment.

Considering the strategy performed in the present study, cell recycle batch fermentation at 30ºC showed to be a promising technique to be applied to xylose conversion to bioethanol using *Scheffersomyces stipitis* strains, since it provided after five recycles, 34.54 g L^{-1} of ethanol with 49%

xylose consumption. The use of 25ºC demonstrated to achieve lower values of ethanol production parameters.

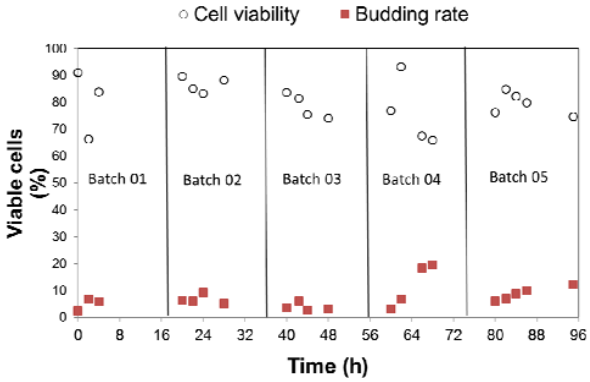

Fig. 6 *S. stipitis* cells viability (%) during five consecutive recycle fermentations at 30ºC using xylose and glucose as substrates

IV. Conclusions

In this work, the use of *Scheffersomyces stipitis*, a xylose-fermenting yeast able to produce ethanol, and the technique of a bioreactor with cell recycle, represented and effective action in order to improve second generation ethanol production.

S. stipitis showed tolerance and growth over the commercial substrate rich in xylose, at 25 and 30ºC. The maximum ethanol concentration was 34.5 g L^{-1} obtained on the 5th recycle cell during fermentation performed at 30°C.

The results are promising and open up possibilities and expectations for hemicellulose hydrolysate use as substrate source by *S. stipitis*.

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