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Increase in Second Generation Ethanol Production by Different Nutritional Conditions from Sugarcane Bagasse Hydrolysate using a *Saccharomyces cerevisiae* Native Strain

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The lignocellulosic materials are an alternative feedstock for ethanol production, because their low cost and high availability. Sugarcane bagasse is an agroindustrial residue that has been identified as a feasible option due to its high sugar content. A second generation process of ethanol production consists of three steps: pre-treatment, enzymatic hydrolysis and fermentation. Two of the most critical aspects during hydrolysate fermentation to obtain high ethanol productivity are: yeast selection and nutrient supplementation. Native yeast strains isolated from extreme environments as sugarcane distilleries could have an easy adaptation to new medium as lignocellulosic biomass hydrolysate that could contains high inhibitors concentrations. The objective of this study was to evaluate different nutrients supplementation ($ZnSO_4$, $MgSO_4$, $MnSO_4$ and KH_2PO_4) in a sugarcane bagasse hydrolysate to increase ethanol productivity during fermentation with a native strain of *Saccharomyces cerevisiae* (202-3). One central composite design with a response surface methodology (RSM) was performed in order to identify the optimal experimental condition. The best results were 0.480 g ethanol g glucose^{-1} ethanol yield and 1.72 g^{-1} L^{-1} h^{-1} ethanol volumetric productivity on a hydrolysate supplemented with 1.0 g L^{-1} KH_2PO_4, 0.05 g L^{-1} $MgSO_4$, 0.01 g L^{-1} $ZnSO_4$ and 0.001 g L^{-1} $MnSO_4$.

1. Introduction

In the last decades, bioethanol has been recognized as an efficient alternative to petrochemical fuels. Renewable resources like biomass are a sustainable feedstock to produce biofuels with a subsequent reduction on environmental impact.Lignocellulosic materials are a renewable source for low-cost ethanol generation, because they are agricultural by-products with a high concentration of cellulose that could be converted in glucose. The use of the whole portion of sugarcane in a sugar refinery included agro-industrial waste named sugarcane bagasse (Andrade, et al., 2014). It is obtained during cane milling, its composition ranges between 19-24% of lignin, 27-32% of hemicellulose, 32 - 44% of cellulose and 4.5 - 9.0% of ashes (Soccol, et al., 2011).To obtain fermentable sugars from lignocellulosic biomass are required two important steps: a pre-treatment and an enzymatic hydrolysis. Pre-treatment stage is applied to facilitate enzyme accessibility to cellulose chains and the subsequent stage to release glucose. In order to obtain ethanol is required a fermentation stage. *Saccharomyces cerevisiae* is the common microorganism in fermentation processes, thus its high tolerance to inhibitors and ethanol concentration, not requirement of oxygen, low optimum pH. Fermentation industry relies on a small fraction of the yeasts diversity (Steensels, et al., 2014), ignoring potential native strains, that due to their evolution and adaptation could reach higher ethanol productions and productivities than current industrial strains. For this reason it is important to evaluate its potential to be used in industrial process.

Though yeasts are versatile microorganisms on the development of fermentation process, they require different elements and micronutrients necessary for an optimum ethanol production. Nitrogen is involved in

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nitrogenized cell compounds formation (protein, amino acids, nucleic acids and metabolites).Nitrogen content is usually quantified by Yeast Assimilable Nitrogen (YAN) which minimal value depends specifically of the strain, but ranges between 120 - 257 mg L^{-1} in the fermentation medium (Aranda, et al., 2011)(Vilanova, et al., 2015). Phosphorous is required in energy generation and metabolism cell, because it is involved on ATP and phosphoric compounds formation. Also cells require a wide range of minerals for their growth and metabolic operation, like magnesium, calcium, manganese, zinc, etc. Zinc has been recognized as an important factor on fermentation, because it acts as an activator in the ethanol dehydrogenase. This enzyme is involved in the conversion of acetaldehyde into ethanol in the Embden-Meyehof-Parnas pathway (Zhao, et al., 2009). Magnesium and manganese are cofactors involved in different metabolic and bioenergetics pathways (Walker, 2004).

Due to the absence of micronutrients and minerals in sugarcane bagasse, this study is focused on investigate the effect of nutrient supplementation on sugarcane hydrolysates, using a native strain of *S.cerevisiae* with the aim to reach higher volumetric productivities with a possible industrial potential.

2. Methods

2.1 Strain

A group of *S.cerevisiae* strain was isolated from a sugarcane distillery in Puerto Lopez, Meta (Colombia). Was made a fermentation study (data not showed) with the aim to isolate the strain with the best performance on the ethanol production and the higher inhibitor tolerance. The selected strain was *S. cerevisiae* (202-3).

2.1 Preparation of sugarcane hydrolysate

The sugarcane bagasse was treated with acid (H_2SO_4) and then the lignin chains on the material became broken, letting the enzymatic treatment arise higher concentrations of glucose in the hydrolysate. Soaking was made with H_2SO_4 2% w/w on the crude bagasse (20% weight of Biomass/Acid Volume) at 60°C for 1 h. The biomass was pressed reducing the acid content until 32% weight of Biomass/Acid Volume. Later was made a pressurized heating at 160°C in a Parr Reactor for 10 min. The obtained solution was neutralized with NH_4OH and then was developed the enzymatic hydrolysis stage with an enzymatic extract (Novozymes ®)for 5 d at 50°C and 120 rpm in a 3 L reactor with an effective volume of 700 mL.The main composition of the hydrolysate used on the fermentation is shown in *(Table 1)*.

Table 1: Characterisation of sugarcane hydrolysate.

Property	Value
Humidity (% w/w)	88.7
Glucose (g L^{-1})	47
Xylose (g L^{-1})	27
pH	4.963
FAN (Free amino nitrogen) (mg L^{-1})	1,146
Acetic Acid (g L^{-1})	5.0
HMF (g L^{-1})	0.03
Furfural (g L^{-1})	0.03

2.2 Inoculum culture preparation

The inoculums of *S.cerevisiae 202-3* was prepared transferring a couple colonies from a solid culture (with composition 20 g L^{-1} glucose, 20 g L^{-1} agar-agar, 10 g L^{-1} yeast extract and 10 g L^{-1} tryptose with distilled water) into a 250 mL Erlenmeyer with 50 mL of propagation medium (with composition 50 g L^{-1} glucose, 5 g L^{-1} tryptose, 3 g L^{-1} malt extract, 3 g L^{-1} yeast extract, 1 g L^{-1} NH_4Cl and 0.4 g L^{-1} KH_2PO_4 with distilled water). The culture was incubated for 16 h at 32°C in an incubator-shaker.

2.3 Fermentation

Fermentation was developed on a 50 mL flask with 40 mL (previously sterilized at 120°C) of sugarcane bagasse hydrolysate (Previously pasteurized at 80°C for 30 min) at 32°C. It was transferred 400µL of concentrated cellular suspension (300mg mL^{-1}), obtaining a 1% v/v inoculum and 1.3 x 10^6 cells by mL of hydrolysate. For the anaerobic condition, was used an airlock with sterilized glycerine. The fermentation was developed for 24-72 h and the sampling was made at 6, 11, 14, 24, 36, 48 and 72 h.

2.4 Experimental design

Experiments were carried out on sugarcane hydrolysates using different nutrients concentrations *(Table 2)* with a native strain of *Saccharomyces cerevisiae (202-3*). A 2^4 full factorial design with two coded levels was used to develop a statistical model for the highest ethanol productivity. A repetition of the experiment was carried out to estimate the experimental error of the data.

Table 2: Minimum and maximum levels of nutrients used in the fermentations.

Variable	Symbol	Minimum Level	Maximum Level
KH_2PO_4	A	0.5 g L^{-1}	1 g L^{-1}
$MgSO_4$	B	0.05 g L^{-1}	0.5 g L^{-1}
$MnSO_4$	C	0.001 g L^{-1}	0.01 g L^{-1}
$ZnSO_4$	D	0.001 g L^{-1}	0.01 g L^{-1}

2.5 Analytical Methods

The glucose, xylose, ethanol, hydroxymethylfurfural, acetic acid and furfural concentrations were quantified by HPLC (High Pressure Liquid Chromatography), using a BioRadAminex® HPX-87H column eluted at 65°C with 5mM H_2SO_4 at a flow rate of 0.6 mL/min and a refractive-index detector at 4 °C. The Free Amino Nitrogen was measured by ninhydrin method reported by (Lie, 1973).

3. Results and discussion

The ethanol production (g L^{-1}) is shown (*Table 3*) for each one of the experimental units. Also are presented the data about productivity (g L^{-1} h^{-1}) at 11 hours (Maximum productivity identified). The evaluation of the more important factors influencing the ethanol productivity at 11h was made through a Pareto chart (*Figure 1*), from the results obtained from the experimental design.

Table 3: Minimum and maximum levels of nutrients used in the fermentations.

Runs	KH_2PO_4 (g L^{-1})	$MgSO_4$ (g L^{-1})	$ZnSO_4$ (g L^{-1})	$MnSO_4$ (g L^{-1})	Ethanol (g L^{-1}) at 11h	Ethanol (g L^{-1}) at 24h	Ethanol Productivity at 11h (g $L^{-1}h^{-1}$)
1	0.5	0.05	0.001	0.001	15.67	23.00	1.425
2	0.5	0.05	0.001	0.01	15.60	23.07	1.420
3	0.5	0.05	0.01	0.001	16.57	22.84	1.506
4	0.5	0.05	0.01	0.01	15.72	22.98	1.430
5	1	0.05	0.001	0.001	17.59	23.46	1.599
6	1	0.05	0.001	0.01	17.40	23.06	1.582
7	1	0.05	0.01	0.001	17.82	23.07	1.612
8	1	0.05	0.01	0.01	18.42	22.82	1.675
9	0.5	0.5	0.001	0.001	13.65	22.74	1.240
10	0.5	0.5	0.001	0.01	16.19	23.31	1.471
11	0.5	0.5	0.01	0.001	15.34	23.03	1.394
12	0.5	0.5	0.01	0.01	15.88	22.91	1.443
13	1	0.5	0.001	0.001	18.54	22.92	1.685
14	1	0.5	0.001	0.01	16.69	22.91	1.517
15	1	0.5	0.01	0.001	18.16	23.15	1.650
16	1	0.5	0.01	0.01	17.80	23.03	1.620

The Pareto chart shows that the most important factor influencing the ethanol productivity at 11 h of fermentation is KH_2PO_4, next to $ZnSO_4$ and the interactions between the KH_2PO_4 with the other compounds added. Also it is possible to perceive a positive effect on the ethanol productivity at 11 h with the addition of KH_2PO_4 and $ZnSO_4$. This is explained through the metabolic pathway of conversion of glucose on ethanol. Phosphates are included in the pathway as ATP and ATP-enzymes for the glucose transformation. Zinc as phosphates, are too directly involved in ethanol generation (Ethanol dehydrogenase cofactor) (Walker, 2004), therefore medium's supplementation with this compounds may achieve a higher productivity on the process.

With the data obtained from the experimental design, was created a model to find the optimum condition of supplementation. The response variable in the model was the volumetric productivity (Q_{Eth}) at 11h. The model obtained is shown on Eq (1), where A is referring to KH_2PO_4 (g L^{-1}), B to $MgSO_4$ (g L^{-1}), C to $MnSO_4$ (g L^{-1}) and D to $ZnSO_4$ (g L^{-1}).

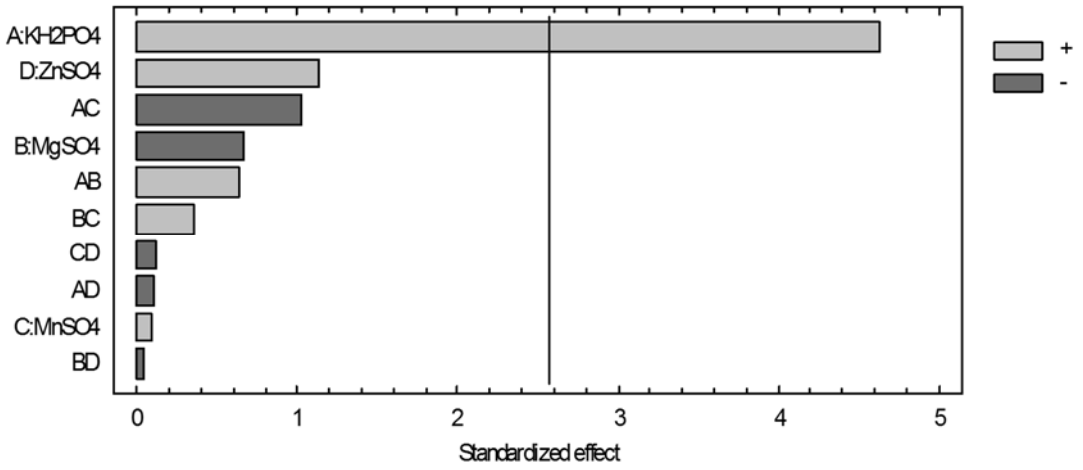

Figure 1: Pareto Chart improving the principal effects of compound supplemented in the ethanol volumetric productivity (g $L^{-1}h^{-1}$).

The model adjust in 83.2% (R-squared), so is not possible to perfectly predict the supplementation effect on the fermentation, but allows to recognize the positive or negative influence of each one of the compounds added in the medium.

$$Q_{Eth}(g\ L^{-1}h^{-1}) = 1.165 + 0.457A + 0.250AB + 20.060AC - 2.079AD - 0.290B + 7.790BC - 0.807BD + 14.109C - 136.72CD + 8.070D \quad (1)$$

With the model obtained and the Pareto Chart was developed a response surface (*Figure 2*) with the principal compounds affecting ethanol productivity (KH_2PO_4 and $ZnSO_4$). The compounds with a less importance on ethanol productivity are $MnSO_4$ and $MgSO_4$ and for this reason was selected the concentrations, where were identified the maximum productivities ($MgSO_4$ (0.05 g L^{-1}), $MnSO_4$ (0.001 g L^{-1})). Also, was obtained the response surface for higher ethanol productions (g L^{-1}) in base to the KH_2PO_4 and $ZnSO_4$ concentration (*Figure 2*).

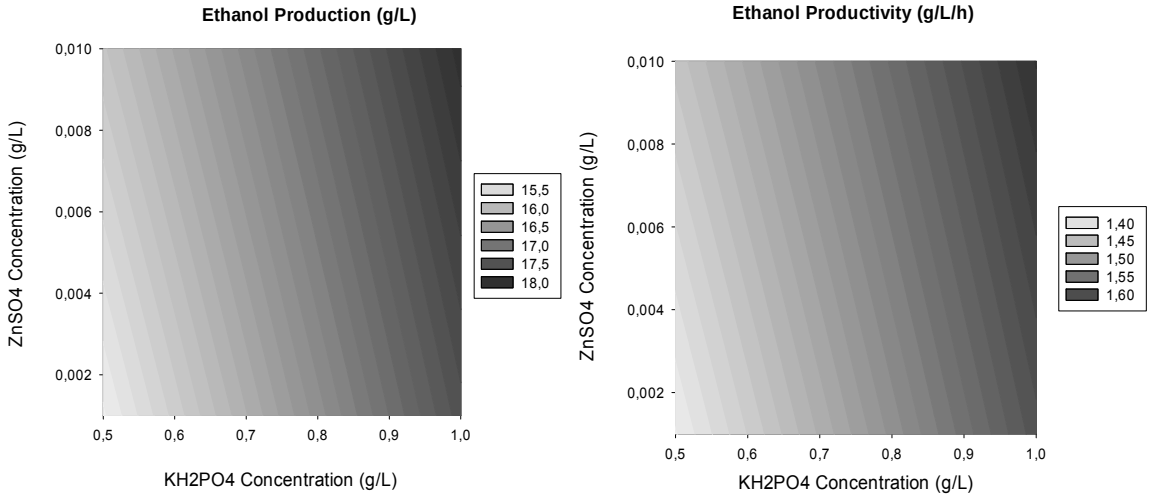

Figure 2: Down: Response surface for Ethanol Volumetric Productivity (g L^{-1} h^{-1}) in base of $ZnSO_4$ and KH_2PO_4 concentration. Up: Response surface for Ethanol Production (g L^{-1}) in base of $ZnSO_4$ and KH_2PO_4 concentration.

It is important to recognize that the selected strain of *Saccharomyces cerevisiae* is able to consume the 98% of initial glucose at 34 h in hydrolysate without supplementation, however adding the enricher compounds to

the broth the strain is are able to consume the 98% of initial glucose at the 24 h. In this way, the strain is able to consume 58 – 87% of initial glucose at 11 h depending on the factors added to the broth.
With the experimental optimal supplementation obtained from the response surface - $MgSO_4$ (0.05 g L^{-1}), $MnSO_4$ (0.001 g L^{-1}), KH_2PO_4 (1.0 g L^{-1}) and $ZnSO_4$ (0.010 g L^{-1}) - was developed a fermentation to analyze the differences between the supplementation and the single hydrolysate. The experimental evaluation of fermentation was made through the volumetric productivity of ethanol at different times (*Figure 3*), where is possible to observe the increasing in the volumetric productivity at 11 h in the supplemented medium.

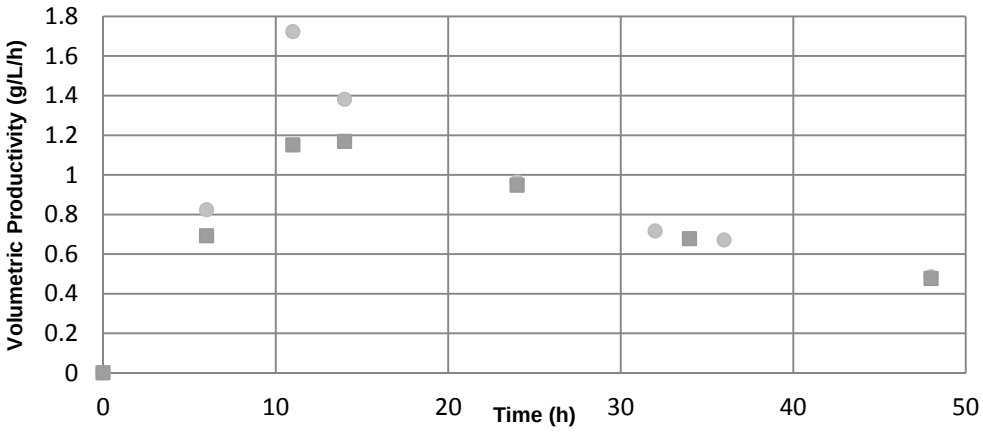

Figure 3: Experimental evaluation of ethanol Volumetric Productivity (g L^{-1} h^{-1}) in the time for the optimized medium (circles) and the broth without supplementation (squares).

The requirement of supplementation is identified with KH_2PO_4 and $ZnSO_4$ in hydrolysates for the increasing of the ethanol volumetric productivity, due to the absence of compounds rich in zinc and phosphorous in lignocellulosic materials as the sugarcane bagasse. Nitrogen enrichment (More important than phosphorous and Zinc) is supplied in the pretreatment, because the neutralization with NH_4OH enriches the medium in nitrogen (Evident on the FAN value).
There are many requirements for *Saccharomyces cerevisiae*. strains to be improved at industrial scale like ethanol productivity, inhibitor tolerance, ethanol yield and inexpensive medium formulations. Different authors consider as necessary in the ethanol biotechnological production, ethanol productivity higher than 1 g L^{-1} h^{-1} (Dien, et al., 2003)(Hanh-Hägerdal, et al., 2007). All industrial strains of *S. cerevisiae* are able to generate ethanol at higher yields (Superior to 90%) (Hanh-Hägerdal, et al., 2007) then the strain evaluation for batch processes is already done in terms of maximum volumetric productivity.
The strain of *S.cerevisiae* 202-3 analyzed in this study is able to reach elevate yields of ethanol (98 - 99%) at 24 hours and higher productivities (approx. 1.1 g L^{-1} h^{-1}), but supplementation with KH_2PO_4 and $ZnSO_4$ in hydrolysates, allows the improvement of the strain at industrial scale due to the higher volumetric productivity achieved (approx. 1.72 g L^{-1} h^{-1}). The volumetric productivity is superior to many of the maximum volumetric productivities reported on the literature for industrial strains of *S. cerevisiae* and other recombinant strains (0.97 – 1.16g L^{-1} h^{-1}) (Bothast, et al., 1999) and even superior to other optimized mediums (Martín, et al., 2002)(Singh & Bishnoi, 2013).
The inhibitor tolerance is an important factor on strain selection, due to the elevate generation of by-products as acetic acid, phenolic compounds and furfurals on pretreatment stage that could inhibit fermentation(Taherzadeh, et al., 2011) at concentrations of 1.0 g L^{-1} of furaldehydes and 5.0 g L^{-1} of aliphatic acids (Martín, 2007) . However the fermentative capacity (yield of ethanol) of this strain is greater than other reported with *S.cerevisiae* in rich medium without inhibitors (Paciello, et al., 2014 and Putra, et al., 2014). Selection of inhibitors resistant strains avoid the detoxification stages on hydrolysates (Dussán, et al., 2014).Add to the higher productivities achieved, the strain is able to ferment with elevate concentrations of inhibitors (5.0 g L^{-1} Acetic Acid, 0.03 g L^{-1} HMF, 0.03 g L^{-1} Furfural) generated in the hydrolysis and pre-treatment stages.

4. Conclusion

The sugarcane hydrolysates are media rich on glucose that allows elevate productions of ethanol through the use of strains of *Saccharomyces cerevisiae*, however is necessary the supplementation with different compounds with the aim to achieve higher volumetric productivities and then make rentable a fermentation

process with the selected strain. For the improved hydrolysate is required to supplement with KH_2PO_4 and $ZnSO_4$ principally (1 g L^{-1} and 0.01 g L^{-1}) for an native strain (*S. cerevisiae* 202-3) able to reach a volumetric productivity (1.72 g L^{-1} h^{-1}) at 11 h with an glucose consume of 98% at 24 h and an ethanol yield of 0.480 g ethanol g $glucose^{-1}$.

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References

Andrade, R. Maugeri, F. Maciel Filho,R. Carvalho da Costa, A. 2014. Alcoholic fermentation from sugarcane molasses and enzymatic hydrolysates: modeling and sensitivity analysis. Chemical Engineering Transactions, 37, 349-354.

Aranda, A., Mantallana, E. del Olmo, M. I., 2011.*Saccharomyces* Yeasts I. Primary Fermentation. In: E. Mantallana, ed. Molecular Wine Microbiology. London, UK.ElsevierAcademic Press, 1-31.

Bothast, R. J., Nichols, N. N. Dien, B. S., 1999. Fermentations with New Recombinant Organisms. Biotechnology Prog, 15, 867-875.

Dien, B. S., Cotta, M. A. Jeffries, T. W., 2003. Bacteria engineered for fuel ethanol production: Current Status. Applied Microbiology Biotechnology, 63, 258-266.

Dussán, K. J., Silva, D. D. V., Moraes, Elisângela J. C., Arruda, P. V., Felipe M. G. A. Dilute – acid hydrolysis of cellulose to glucose from sugarcane bagasse. Chemical Engineering Transactions Vol. 38. 433-438.

Hanh-Hägerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins I., Gorwa-Grauslund.M. F. 2007. Towards industrial pentose-fermenting yeast strains. Applied Microbiology Biotechnology, 74, 937-953.

Lie, S., 1973. The EBC Ninhydrin method for determination of free alpha amino nitrogens. Journal Institute Brewing, 79, 7-41.

Martín, C., Galbe, M., Fredrik Wahlbom C., Hahn-Hägerdal B., Jönsson L. J. 2002.Ethanol production from enzymatic hydrolysates of sugarcanne bagasse using recombinant xylose-utilising *Saccharomyces cerevisiae*. Enzyme and Microbial Technology, 31, 274-282.

Martín, C. Marcet, M. Almazán, O. Jönsson, L. J. Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresource Technology, 88, 1767-1773.

Paciello, L. Parascandola, P. Landi, C.2014. Auxotrophic *Saccharomyces cerevisiae* CEN.PK strains as new performers in etanol production. Chemical Engineering Transactions,38, 463-468.

Putra, M.D. Sulieman,A.K. Zeinelabdeen, M.A.2014. Utilization of pitted dates of the production of highly concentrated fructose syrups by *Saccharomyces cerevisiae*. Chemical Engineering Transactions, 38, 397-402

Singh, A. Bishnoi, N., 2013. Ethanol production from pretreated wheat straw hydrolysate by *Saccharomyces cerevisiae* via sequential statistical optimization. Industrial Crops and Products, 41, 221-226.

Soccol, C. R. Faraco V., Karp S., Vandenberghe L. P. S., Thomaz-Soccol V., Woiciechowski A., Pandey A. 2011. Lignocellulosic Bioethanol: Current Status and Future Perspectives. In: A. Pandey A., Larroche, C., Ricke Steven C., Dussap C., Gnasounou, E. edits. Biofuels: Alternative Feedstocks and Conversion Process. London, UK. Elsevier Academic Press, 101-122.

Steensels, J. Snoek T., Meersman E., Picca Nicolino M., Voordeckers K, Verstrepen K. J. 2014. Improving industrial yeasts strains: Exploiting natural and artificial diversity. Federation of European Microbiological Societies Microbiology Review, 1-49.

Taherzadeh, M. J., Karimi, K. 2011.Fermentation inhibitors in Ethanol Processes and different strategies to reduce their effects.In: A. Pandey, A., Larroche, C., Ricke Steven C., Dussap C., Gnasounou, E. edits. Biofuels: Alternative Feedstocks and Conversion Process. London, UK. Elsevier Academic Press, 287-311.

Vilanova, M., Pretorius, I. S. Henscheke, P. A., 2015. Influence of Diammonium Phosphate Addition to Fermentation on Wine Biologicals. In: V. Preedy, ed. Processing and Impact on Active Components in Food. London: Elsevier Academic Press, 471-481.

Walker, G. M., 2004. Metals in Yeast Fermentation Process. Advances in Applied Microbiology, 54, 197-225.

Zhao, X., Xue C., Ge XM., Yuan WJ., Wang JY., Bai FW. 2009. Impact of zinc supplementation on the improvement of ethanol tolerance and yield of self-flocculating yeast in continuous ethanol fermentation. Journal of Biotechnology, 139, 55-60.