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Valorization of sunflower meal through the production of ethanol from the hemicellulosic fraction

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ABSTRACT
Sunflower is among the major oil seeds crop grown in the world and the by-products generated during the seeds processing represent an attractive source of lignocellulosic biomass for bioprocesses. The conversion of lignocellulosic fibers into fermentable sugars has been considered as a promising alternative to increase the demand for ethanol. The present study aimed to establish the fermentation conditions for ethanol production by *Scheffersomyces stipitis* ATCC 58376 in sunflower meal hemicellulosic hydrolysate, through a 2^3 CCRD (Central Composite Rotational Design) factorial design. Under the selected conditions (pH 5.25, 29 ºC and 198 rpm) the final ethanol concentration was 13.92 g L^{-1} and the ethanol yield was 0.49 g g^{-1}.

Palavras-chave:
subproduto
biomassa
xilose
biocombustível
otimização

Valorização do farelo de girassol através da produção de etanol a partir da fração hemicelulósica

RESUMO
O girassol está entre as principais oleaginosas cultivadas no mundo e os subprodutos gerados durante o processamento das sementes representam uma fonte atraente de biomassa lignocelulósica para bioprocessos. A conversão de fibras lignocelulósicas em açúcares fermentáveis tem sido considerada uma alternativa promissora para aumentar a demanda de etanol. O presente estudo teve, como objetivo, estabelecer as condições de fermentação para a produção de etanol por *Scheffersomyces stipitis* ATCC 58376 em hidrolisado hemicelulósico de farelo de girassol através de um planejamento fatorial DCCR 2^3. Sob as condições selecionadas (pH 5,25, 29 ºC e 198 rpm) a concentração final de etanol foi 13,92 g L^{-1} e o rendimento 0,49 g g^{-1}.

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Introduction

Among the oil crops, sunflower (*Helianthus annuus*) is the one that presents the highest expansion rate in the world. According to the United States Department of Agriculture (USDA, 2015), the world production of sunflower seeds reached 35.8 million MT (metric tons) in the 2012/13 marketing year and 42.7 million MT in 2013/14, representing an increase of 19%. For 2014/15, the global production is expected to be 39.8 MT. Sunflower meal, the by-product of the oil extraction process, has been generally used as food supplement for non-ruminants (Hernández et al., 2011) and as biomass for power generation (Raclavska et al., 2011).

Lignocellulosic biomass is typically nonedible plant material composed primarily of the polysaccharides cellulose and hemicellulose. Lignin, a phenolic polymer that provides structural strength to the plant, is the third major component (Sluiter et al., 2010).

The economic feasibility of ethanol production from lignocellulosic materials lies on the efficient use of their sugar content. This implies not only the glucose obtained from the cellulose, but also the sugars released from the hemicellulose (Díaz et al., 2009). The dilute-acid hydrolysis pre-treatment is commonly used to separate hemicellulose and the resulting hemicellulosic hydrolysate can also be fermented to produce ethanol (Canilha et al., 2012).

The yeast *Scheffersomyces stipitis,* formerly known as *Pichia stipitis*, has a natural ability to convert hemicellulose-derived sugars into ethanol (Krahulec et al., 2012, Scordia et al., 2012). However, its fermentation efficiency is related to nutritional factors such as temperature, pH and oxygen supply, and toxic factors present in the hemicellulose hydrolysate (Du Preez et al., 1986; Prior et al., 1989), as well as the tolerance to ethanol and lignocellulose-derived inhibitors (Bellido et al., 2011).

Recent studies have presented sunflower biomass as a novel source of sugars from cellulose and hemicellulose (Camargo et al., 2014a) and for ethanol production by simultaneous saccharification and fermentation (SSF) (Camargo et al., 2014b). Although there are many studies in the literature on the physiology of *S. stipitis* and its potential to produce ethanol from xylose, reports on its performance in hemicellulosic hydrolysates derived sunflower by-products are still limited. Thus, the present study aimed at exploring the biotechnological potential of sunflower meal for ethanol production by *S. stipitis* from the hemicellulosic hydrolysate.

Material and Methods

Characterization of sunflower meal (Caramuru Alimentos, Itumbiara-GO, Brazil), dilute acid hydrolysis of the hemicellulosic fraction (6% w v^{-1} H_2SO_4, 121 °C, 20 min) and hydrolysate detoxification (pH adjustment followed by adsorption with activated charcoal) were performed according to Camargo & Sene (2014).

Scheffersomyces (*Pichia*) *stipitis* ATCC 58376 was maintained in YMA agar slants (3 g L^{-1} malt extract, 3 g L^{-1} yeast extract, 5 g L^{-1} peptone, 10 g L^{-1} dextrose, 20 g L^{-1} agar) at 4 °C. Cells were pre-adapted to the inhibitors by growing the inoculum in the hemicellulosic hydrolysate (45.56 g L^{-1} xylose) supplemented with 3 g L^{-1} malt extract, 3 g L^{-1} yeast extract and 5 g L^{-1} peptone, in Erlenmeyer flasks, at 30 °C, 200 rpm, for 16 h. The initial cell concentration was 1 g L^{-1} estimated by a standard curve (dry cell mass vs. $O.D._{600\ nm}$).

Fermentations were conducted in shaker in 125-mL Erlenmeyer flasks with 50 mL of sunflower meal hemicellulosic hydrolysate (45.56 g L^{-1} xylose), supplemented with the nutrients mentioned above, for 84 h, according to a 2^3 - CCRD (Central Composite Rotational Design) factorial scheme, with 8 major tests, 6 axial and 3 central, totalizing 17 tests in different conditions of pH, temperature and agitation (Table 1). The software Statistica 8.0 was used for data analysis.

Glucose, xylose, arabinose, acetic acid and ethanol concentrations were determined in a liquid chromatograph with refractive index detector, using a Phenomenex Rezex ROA Organic Acid H+ (8%) 150 x 7.8 mm column, eluent 0.005 mol L^{-1} H_2SO_4, flow rate of 0.6 mL min^{-1} and oven temperature of 65 °C. Furfural and HMF were analyzed using a Restek Allure C18 5 µm 4.6 x 250 mm column, UV/VIS detector at 276 nm, eluent acetonitrile: water (1:8) with 1% acetic acid, flow rate 0.6 mL min^{-1} at room temperature. Standard curves were prepared with high purity compounds (98-99%, Vetec and Sigma).

Total phenols were assayed according to the Folin Ciocalteu method (Singleton et al., 1999), using a standard curve of vanillin (purity 98%, Synth).

Table 1. Encoded and real values of the independent variables pH, temperature and agitation and the respective levels of variation for the 2^3 CCRD (Central Composite Rotational Design)

Experiment/ test	Independent variables					
	Agitation (rpm)		pH		Temperature (°C)	
	Encoded	Real	Encoded	Real	Encoded	Real
1	-1	100	-1	4.5	-1	25
2	-1	100	1	5.5	-1	25
3	-1	100	-1	4.5	1	35
4	-1	100	1	5.5	1	35
5	1	200	-1	4.5	-1	25
6	1	200	1	5.5	-1	25
7	1	200	-1	4.5	1	35
8	1	200	1	5.5	1	35
9	0	150	-1.68	4.2	0	30
10	0	150	1.68	5.9	0	30
11	0	150	0	5	-1.68	22
12	0	150	0	5	1.68	38
13	-1.68	66	0	5	0	30
14	1.68	234	0	5	0	30
15	0	150	0	5	0	30
16	0	150	0	5	0	30
17	0	150	0	5	0	30

Results and Discussion

Chemical characterization of sunflower meal resulted in the following composition: 32.93% cellulose, 30.90% hemicellulose, 26.62% lignin, 5.05% ash, 27.93% protein and 1.60% lipids.

The sunflower meal hemicellulosic hydrolysate presented the following sugar composition: glucose 8.06 g L^{-1}, xylose 49.93 g L^{-1} and arabinose 8.67 g L^{-1}. Xylose content in sunflower hydrolysate was high, compared to the hemicellulosic hydrolysates obtained from other materials such as sorghum straw, 17.69 g L^{-1} (Sene et al., 2011), sugar cane bagasse,

17.85 g L^{-1} (Marton et al., 2006), rice straw, 18.33 g L^{-1} (Mussatto & Roberto, 2004) and wheat straw, 19.50 g L^{-1} (Canilha et al., 2005).

The inhibitors concentrations (acetic acid 3.56 g L^{-1}, furfural 0.03 g L^{-1}, HMF 0.03 g L^{-1} and total phenols 0.89 g L^{-1}) are within or under the range of values usually found in other sources of hemicellulosic hydrolysates, in general, 1-3.41 g L^{-1} of acetic acid, 0.04-0.26 g L^{-1} of furfural, 0.008-1.56 g L^{-1} of HMF and 2.12-2.23 g L^{-1} of total phenols (Mussatto & Roberto, 2004; Canilha et al., 2005; Marton et al., 2006; Villarreal et al., 2006; Sene et al., 2011).

After detoxification, the sugar composition in the hydrolysate was 45.56 g L^{-1} of xylose, 7.4 g L^{-1} of glucose, 7.82 g L^{-1} of arabinose, with a slight reduction of sugars (8-10%). Acetic acid concentration was reduced by 58% reaching the value of 1.5 g L^{-1}. Total phenols, furfural and HMF were not detected in the detoxified hydrolysate. It has been reported that fermentations of steam exploded wheat straw by *S. stipitis* were completely inhibited by a synergistic effect due to the presence of 1.5 g L^{-1} of acetic acid, 0.15 g L^{-1} of furfural and 0.05 g L^{-1} of HMF (Bellido et al., 2011), the same acetic acid concentration found in this work after detoxification. However, the development of a *P. stipitis* more tolerant to acetic acid and other inhibitory components present in acid hydrolysates is possible through a simple adaptation of cells (Nigam, 2001).

Xylose consumption was strongly influenced by the different conditions employed. The highest xylose consumption was observed in the condition 14 (pH 5, 30 °C and 234 rpm), which was very similar to the xylose consumption at the central point triplicates - treatments 15, 16 and 17 (pH 5, 30 °C and 150 rpm). No xylose assimilation was observed in the conditions 11 (pH 5, 22 °C, 150 rpm) and 12 (pH 5, 38 °C, 150 rpm). Results suggest that the furthest temperature values employed (22 and 38 °C) reduced xylose assimilation, even at intermediate pH and agitation values (pH 5 and 150 rpm), which seemed to be favorable (data not shown).

Glucose uptake was faster in the conditions 6 (pH 5.5, 25 °C, 200 rpm) and 5 (4.5, 25 °C, 200 rpm). Similarly to that observed for xylose, the condition 12 (pH 5, 38 °C, 150 rpm) had strong negative influence on the consumption of glucose. Arabinose concentrations remained unaltered in most of the tests (data not shown), similar to an observation related in a previous work carried out with *S. stipitis* ATCC 58376 grown in sunflower meal hemicellulosic hydrolysate (Camargo & Sene, 2014).

Growth profile seemed to be directly affected by pH, temperature and agitation and their combinations. In general, high growth was related to high agitation (≥ 150rpm) (data not shown). By comparing the growth profile in the conditions 6 (pH 5.5, 25 °C, 200 rpm) and 8 (pH 5.5, 35 °C, 200 rpm), it was observed that the higher temperature in the condition 8 probably affected growth negatively. High growth was also observed at intermediate levels of pH and temperature, i.e., pH 5.0 and 30 °C, regardless of the agitation rate (treatments 13, 14 and the central point repetitions), as well as in the treatment 10 (pH 5.84, 30 °C, 150 rpm).

The maximum ethanol concentration (13.31 g L^{-1}) was obtained in the treatment 14 (pH 5.0, 30 °C and 234 rpm), which coincided with the highest consumption of xylose. Very similar values (average of 13.27 g L^{-1}) were observed in the replicates of the central point - tests 15, 16 and 17 (pH 5.0, 30 °C and 150 rpm), which also presented high xylose assimilation. In the experiment 11 (pH 5.0, 22 °C, 150 rpm) and 12 (pH 5.0, 38 °C, 150 rpm), the lowest and highest temperatures strongly influenced the process, since ethanol was not produced. Low pH values (pH 4.2 and 4.5) and agitation (66 rpm) may also be associated with a low ethanol production.

Similar ethanol yields (0.46-0.50 g g^{-1}) were observed at 72 h in many of the tested conditions, except in the treatments 11 and 12, in which ethanol was not produced and assay 09 and 10 (0.41 and 0.40 g g^{-1}, respectively). In most assays, the ethanol yield decreased slightly until 84 h. In contrast, for treatment 14 ethanol yield was 0.40 and 0.45 g g^{-1} at 72 and 84 h, respectively. In studies using the yeast *S. stipitis* NRRLY-7124 grown in semi-defined medium containing 90 g L^{-1} xylose, an yield of 0.32 g g^{-1} ethanol and productivity of 0.32 g L^{-1} h^{-1} was obtained (Silva et al., 2011). When this same strain was used for ethanol production from sugarcane hemicellulosic hydrolysate, ethanol yield was 0.39 g g^{-1} (Liu et al., 2011).

Thus, the results obtained in the present work were similar or even higher than those mentioned above, which shows that the sunflower hydrolysate may represent an alternative source of xylose to ethanol production. However, volumetric productivity of ethanol was only 0.158 g L^{-1} h^{-1}, which may be increased by using a higher cell density. According to Ding et al. (2009), obtaining quantitative data concerning the impact of inoculation size on yeast growth and metabolism is of great importance for industrial ethanol fermentation process.

Pareto charts for ethanol production (Figure 1) show the standardized effects at a significance level of 0.05 ($p < 0.05$). The quadratic temperature factor produced the most significant effect on the response variable ethanol production during the fermentation process, exerting a negative influence. This effect was even greater (-2023.94) at 72 h and followed by a reduction at 84 h (-1131.66). Linear agitation was the second most significant effect at 24 h (149.7203) and 48 h (83.7899) and the third most significant effect at 72 h (484.07), followed by a reduction at 84 h (418.6215). The interactions among independent variables, agitation x temperature (1 and 3), agitation x pH (1 and 2) and pH x temperature (2 and 3) were significantly higher at 72 h, subsequently decreasing at 84 h. There was no interaction between the variables linear agitation x linear pH at 84 h.

At the fermentation time of 48 h, there was no interaction between variables, while at 24, 72 and 84 h there was interaction between all variables with coefficient of determination of 0.82, 0.82 and 0.77, respectively and p-value less than 0.05. According to the analysis model for the fermentations at times of 24, 72 and 84 h, the F-value calculated was respectively 1.6, 1.47 and 1.27 times the F-tabulated value. In general, as there was a strong interaction between the linear and quadratic variables at 24 and 72 h and reduction of the interactions at 84 h, data of 24 and 72 h were used to construct the response surface graphs (Figure 2).

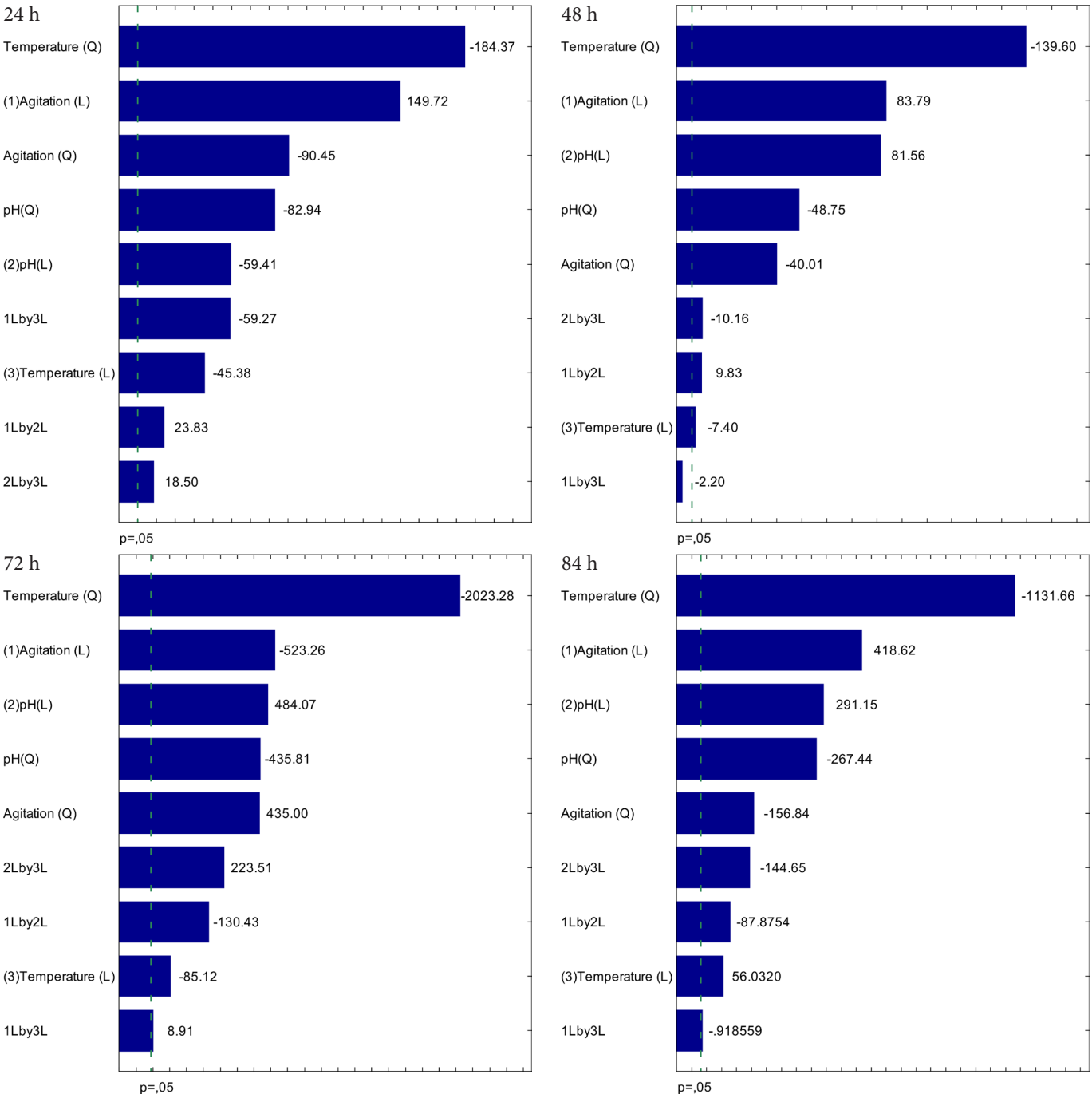

Figure 1. Pareto charts for estimation of the effects of pH, temperature and agitation on the response ethanol production after 24, 48, 72 and 84 h

Figure 2A and 2D show the quadratic effect of pH and agitation on the response ethanol production at 24 and 72 h, respectively, in which it is evident that the greatest contribution to ethanol production occurred at the intermediate levels of pH and agitation. A higher ethanol production was also observed at the intermediate levels of temperature and agitation (Figure 2B and 2E) as well as at intermediate levels of temperature and pH (Figures 2C and 2F). Thus, by evaluating all the data together, it was possible to conclude that the production of ethanol was favored at pH values near 5, agitation between 180-200 rpm and temperature near 30 °C.

A model is considered adequate and close to the optimization when it presents itself as quadratic model. Adequate models were obtained at 72 h, when the variables showed the greatest influences on ethanol production.

Based on the results obtained after the completion of the design (2^3 CCRD), all responses were optimized simultaneously, considering the desirable values for each one. By the interpolation of the results obtained at 72 h, it was possible to verify that the optimal conditions corresponded to pH of 5.25, temperature of 29.0 °C and agitation of 198 rpm. The fermentations carried out in triplicate at the optimized conditions (Figure 3) led to a final ethanol concentration of 13.92 g L^{-1}, which corresponded to an ethanol yield of 0.49 g g^{-1} and an efficiency of 96% compared to the theoretical yield. At this condition, ethanol production increased by 4.38% and volumetric ethanol productivity increased by 4.65% compared to the treatment 14.

Although the favorable conditions for the ethanol production by *S. stipitis* ATCC 58376 in sunflower biomass

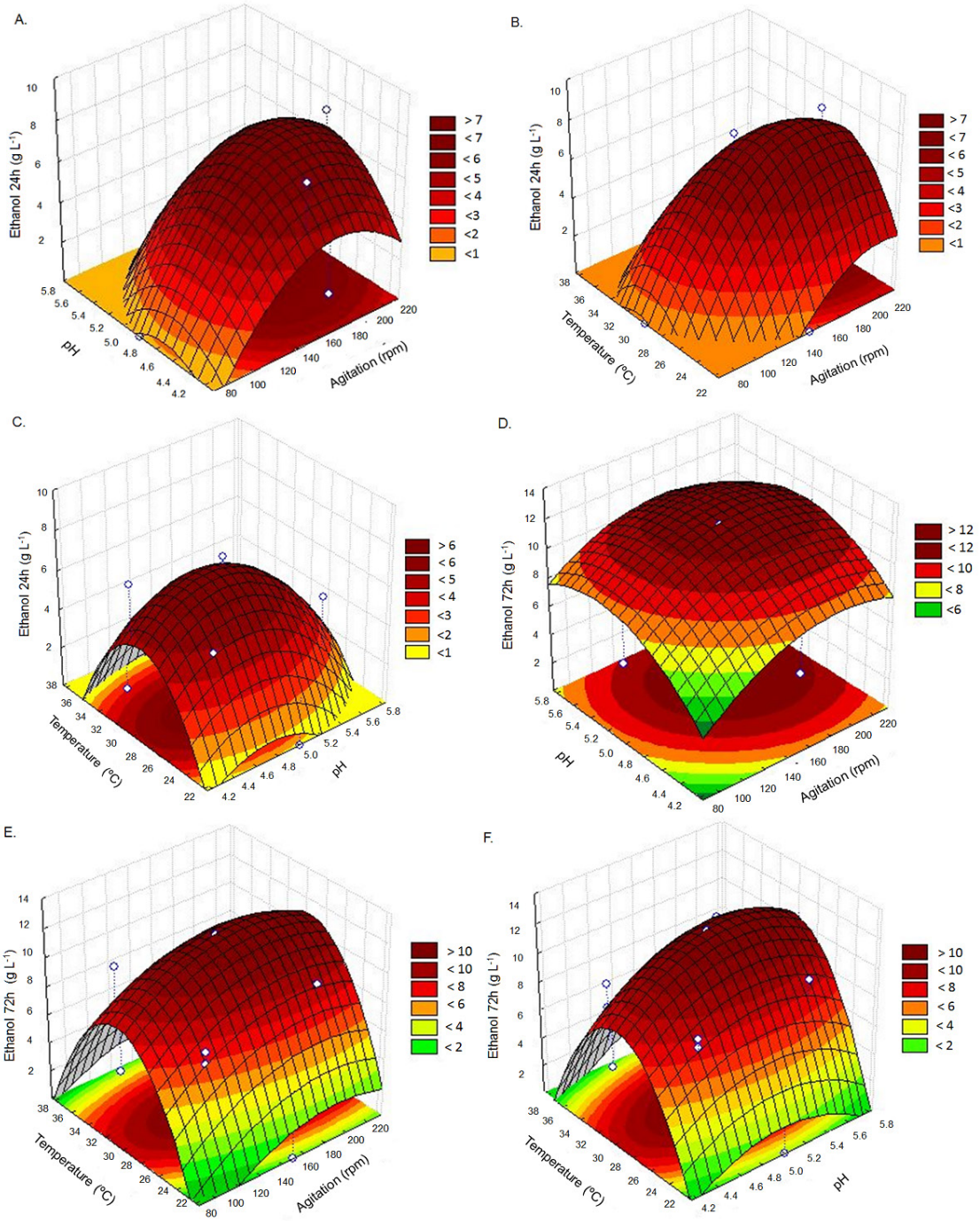

Figure 2. Surface response graphs (pH x agitation, temperature x agitation and pH x temperature) for ethanol production after 24 (ABC) and 72 h (DEF)

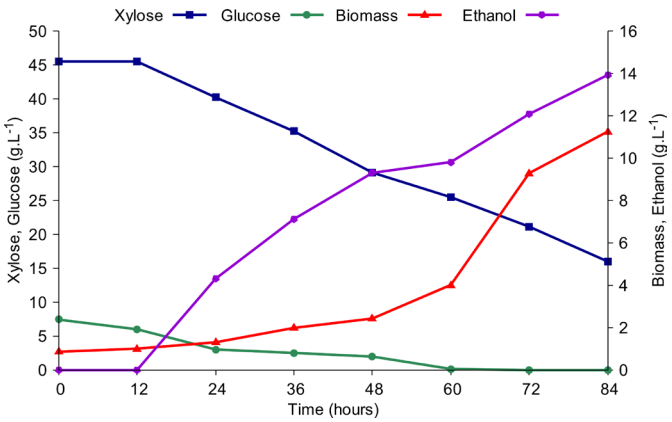

Figure 3. Kinetic profiles of fermentation performed at the optimized conditions

hemicellulosic hydrolysate have been clearly evidenced, the low cell concentration (1 g L^{-1}) initially used as inoculum may have been responsible for the low productivity. High cell-density fermentation can improve the productivity of final products and shorten fermentation time (Liu, 2012). High cell density of *Saccharomyces cerevisiae* strains was proven to provide effective fermentation at high sugar concentrations while mitigating some inhibitory effects of softwood hydrolysates (Kapu et al., 2013).

Conclusions

1. The use of pre-adapted cells to the hydrolysate as inoculum along with the optimization of conditions through experimental design, demonstrated that the sunflower meal

hemicellulosic hydrolysate is feasible for ethanol production by *S. stipitis* ATCC.

2. The sunflower meal hemicellulose hydrolysate presented the advantage of having high sugar concentration, which eliminates the need for a concentration step, resulting in savings in the process of ethanol production, a fuel of great importance for the Brazilian economy.

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