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OPTIMIZATION OF BIOETHANOL PRODUCTION FROM PRICKLY PEAR OF *Opuntia ficus-indica* AT HIGH TEMPERATURES

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Abstract

The cost of saccharine and starchy biomasses represents approximately 60% of first-generation bioethanol production costs. Inputs, seeds, crop irrigation, and crop transportation are important energy factors to consider. To find alternative substrates to costly and food competing biomasses, we explored an agro-biological resource that is drought resistant and tolerant to a wide range of soil and climatic conditions, namely: prickly pear (*Opuntia ficus-indica*).

This work aims to optimize the production of first-generation bioethanol by *Kluyveromyces marxianus* YMEK23, a thermoresistant yeast strain, from prickly pear juice; a substrate rich in sugars (98g L^{-1}). The use of the Box-Behnken experimental design showed that the fermentation temperature and the medium pH are the main parameters influencing ethanol production. The impact of these factors was modeled in a second-degree polynomial equation. The results showed that the maximum amount of ethanol produced was 41 g L^{-1} obtained at 37°C and a pH of 5. However, supplementing nitrogen has a limited impact on ethanol production.

The kinetics of batch fermentation under optimum conditions showed a very active fermentation metabolism of *K. marxianus* on this substrate, translated by an early and exponential production of ethanol as well as a rapid consumption of sugars. The maximum amount of ethanol 41 g L^{-1} was reached after only 16 hours of fermentation.

The high yield of ethanol obtained 0.43 g g^{-1} makes prickly pear biomass an attractive and economical substrate for the production of bioethanol compared with the conventional substrates currently used by the biofuel industry.

Keywords: bioethanol, Box-Behnken experimental design, *Kluyveromyces marxianus*, prickly pear, thermoresistant yeast

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1. Introduction

The increasing consumption of fossil fuels combined with the current ecological and environmental issues represent significant global challenges. To address these challenges, bioethanol constitutes a renewable and environmentally friendly fuel that can improve air quality, lower greenhouse gas emissions, and promote domestic rural economies (Börjesson, 2009; Nigam and Singh, 2011). Although first-generation bioethanol, which is currently the most available biofuel in the market (Vimmerstedt et al., 2012), has a well mastered industrial process, the cost of raw material represents about 60% of its production cost. The technology of second-generation bioethanol has significantly improved. However, its commercialization is still limited by its high production costs compared with fossil fuels (Chen and Fu, 2016; Thangavelu et al., 2016).

With increasing focus on the utilization of inexpensive and non-food competing biomass for bioethanol production, the exploitation of cactus pear using a biorefinery strategy by integrating the extraction of value-added chemicals and the production of fuel appears to be a promising approach. Indeed, *Opuntia ficus-indica*, drought-resistant, and widespread biomass can tolerate a wide range of edaphic and climatic conditions using the crassulacean

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acid metabolism (Nefzaoui and Ben Salem, 2002; Stintzing and Carle, 2005). Its adaptation mechanism enables it to produce up to 50 tons of dry matter per hectare per year (Inglese et al., 2002; Nobel, 2002). The ever increasing interest in *Opuntia ficus-indica* is due to its ecological, environmental, and socio-economic impacts such as the fight against erosion, desertification, and the production of fodder fruit (Bouzoubaâ et al., 2014).

Many countries are investing in the cultivation of cactus for various reasons. For example, in Morocco, the culture of *Opuntia* occupies a large area (150 000 hectares) that is experiencing a remarkable increase due to the efforts of a new strategy for agricultural development concretized by the Green Morocco Plan. Currently, the economic importance of this biomass lies in the quantity of oil contained in its seeds that is subject to high commercial transactions. The remainder of the plant remains under-utilized and gets transformed into juice, syrup, jam and, fodder for livestock.

This study proposes to evaluate the technical feasibility of high-temperature bioethanol production from the prickly pears fruit juice, a crop from drought regions. Moreover, cactus plant cultivation does not require fertile land or irrigation and does not impinge on food availability.

We adopted the Response Surface Methodology (RSM) to quantitatively determine the impact of fermentation parameters on ethanol production from prickly pear juice. Three independent variables were optimized as affecting ethanol production: temperature, pH and, nitrogen supplementation dose. The experiments were designed using a Box-Behnken design.

The fermentation at elevated temperatures in warm climate regions like Morocco will reduce cooling costs, lower the risk of microbial contamination (Abdel-Banat et al., 2010; Fonseca et al., 2008) and prevent enzyme feed-back inhibition by sugar and oligosaccharides produced (Fox et al., 2012) leading to increased overall production yield. The application of such a method requires well-selected yeast strains (Castro and Roberto, 2014; Faga et al., 2010). For the study, we used thermotolerant yeast *Kluyveromyces marxianus* YMEK23. This specie has a higher growth rate and can utilize a greater range of sugars, such as cellobiose, xylose, and arabinose (Lane and Morrissey, 2010), and is known to produce ethanol at temperatures above 40°C while it can reach a maximum growth temperature of 52°C (Banat and Marchant, 1995; Nonklang et al., 2008).

2. Material and methods

2.1. Isolation and yeast strain identification

We isolated the yeast strains used for ethanol production from prickly pears fruit. Their identification was achieved by PCR using ITS1 and ITS4 rDNA specific primers with MyTaq kit (Bioline, France) and their sequencing by the Sanger method (Genetic Analyzer ABI 3130xl).

2.2. Preparation of prickly pear juice

The prickly pear fruits used are the Moussa cultivar variety, belonging to *Opuntia ficus indica* species, obtained from the Aït Bâamrane region in the south of Morocco. These fruits are peeled, pressed, and then filtered to remove the seeds. We directly used the obtained juice as the carbon source in all experiments.

2.3. Experimental design and statistics

We investigated the analytical responses using a Box–Behnken statistical experimental design based on a 3^3 factorial design. We replicated the central run 3 times creating 15 experiments and optimizing every experimental response.

The process of optimizing the model required the evaluation of the response of the statistically modeled combinations, the estimation of the coefficients by fitting the experimental data to the response function, the prediction of fitted model response, and the verification of the overall model adequacy. To keep the effects of uncontrolled factors biasing the response at a minimum level, the order at which the experiments were conducted was randomized.

From preliminary experiments, we identified three independent variables as affecting ethanol production. To quantitatively determine the effect of each parameter on ethanol production, we selected the independent variables (i.e. temperature (X_1), pH (X_2), and nitrogen supplementation dose (X_3)). Ethanol concentration (Y, g L^{-1}), was the response (i.e. dependent variable). As shown in Table 1, we coded the variable levels X_i as x_i as per the following equation $X_i = (x_i . \Delta x_j) + X'_i$. Where x_i is the coded value for the i^{th}, Δx_j is the variation of the real value corresponding to one unit of the coded variable, and Xi' is the real value for the i^{th} variable corresponding to coded value 0. Coded values -1 and 1 are the lower and the upper limit of the independent variable, respectively.

Table 1. Factor Variation Intervals

Independent factors	Unit	Levels		
		Low (-1)	Middle (0)	High (+1)
Temperature, X_1	°C	37	41	45
pH, X_2	-	3.5	5.0	6.5
Nitrogen supplementation, X_3	g L^{-1}	0.0	0.5	1

The experimental designs of the coded (X_i) and actual levels of variables are shown in Table 2. The response function Y is related to the variables (X_i, i = 1, 2, 3) by a second-degree polynomial equation using the least-squares method (Eq. 1):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + \varepsilon \quad (1)$$

where Y was the analytical response; b_0 (constant term), b_1, b_2, b_3 (linear effects), b_{11}, b_{22}, b_{33} (quadratic effects), b_{12}, b_{13}, b_{23} (interaction effects), and ε error term.

The regression and graphical analysis of data were performed with the commercial software JMP (version 13). The coefficient of determination R^2 expressed the adequacy of the statistical model whose statistical significance was verified by an F-test (ANOVA) at a 5% significance level. A student's t-test was used to determine the coefficient's statistical significance. The regression model included only statistically significant coefficients (i.e. p-value < 0.05). Numerical and graphical analysis based on response surface and desirability function criteria determined the optimum processing conditions.

2.4. Batch fermentation

The optimization of ethanol production was carried out in 100 mL flasks containing 30 mL of prickly pears juice, sterilized by autoclaving for 15 minutes at 110°C. The medium was inoculated with 6×10^8 yeast cells/mL of *K. marxianus* YMEK23 and incubated for 24 hours. Stirring was maintained at 450 rpm. In each experiment, the temperature, pH, and nitrogen supplementation were chosen according to the Box-Behnken design matrix as shown in Table 2.

Table 2. Box–Behnken design matrix of the coded and actual variables

Run	Coded values			Real values		
	x_1	x_2	x_3	X_1	X_2	X_3
1	0	0	0	41	5	0.5
2	-1	0	-1	37	5	0
3	-1	0	1	37	5	1
4	0	1	-1	41	6.5	0
5	1	0	-1	45	5	0
6	0	0	0	41	5	0.5
7	0	-1	-1	41	3.5	0
8	0	-1	1	41	3.5	1
9	1	0	1	45	5	1
10	1	1	0	45	6.5	0.5
11	1	-1	0	45	3.5	0.5
12	0	1	1	41	6.5	1
13	-1	1	0	37	6.5	0.5
14	0	0	0	41	5	0.5
15	-1	-1	0	37	3.5	0.5

2.5. Batch fermentation kinetics

The batch fermentation kinetics were performed at 37°C in a fermenter Bioflo (New Brunswick Scientific, NJ, USA), containing 1.3 litres of prickly pear juice and inoculated with 6×10^8 yeast cells/mL. Stirring was kept constant at 450 rpm. Samples were taken every 4 hours to evaluate biomass and ethanol productions and residual sugar.

2.6. Analytical methods

The soluble solid content of prickly pear cactus juice was measured with a refractometer at 20°C and expressed in °Brix. The reducing sugars were quantified by 3.5-dinitrosalicylic acid colorimetric assay (Miller, 1969). The standard curve was in the range of 0.1–1 (g L^{-1}) glucose solution (R^2 = 0.987). The nitrogen content analysis is carried out using the Kjeldahl method. The ethanol content was determined by previously described techniques (Jamai et al., 2001). The titratable acidity was determined by citric acid. A 5 mL aliquot of cactus pear juice was titrated with 0.01 mol.L^{-1} NaOH (Camara et al., 1994).

3. Results and discussion

3.1. Isolation and yeast strain identification

The raw prickly pear juice analysis showed 14.63°Brix. This value is identical to those indicated by (Saenz, 2000) who reported a range of 12°-15°Brix for *Opuntia ficus indica*. However, the obtained value is higher than the 6.2°-12.6°Brix range reported by (El Kharrassi et al., 2016). The sugar content is strongly influenced by cultural practices (fertilization, irrigation) and environmental factors as the sugar concentration can be improved by nitrogen fertilization (Potgieter and Mkhari, 2002). The content of reducing sugars in our prickly pear juice was 98 ± 1.67 g L^{-1}, the nitrogen content was 53 mg / 100 mg, and the pH measured was 4.5. Such a low pH is important in fruit juices since it inhibits pathogens' growth (Mert, 2010). The total acidity analyzed in this study expressed as w/w percentage of monohydrate citric acid is 0.07 %, which is in alignment with values reported in the literature (0.05 - 0.18 %) (El Kharrassi et al., 2016; Mert, 2010; Potgieter and Mkhari, 2002; Saenz, 2000; Sepulveda, 1998).

3.2. Experimental design

Among several statistical experimental design methods that are currently employed in bioprocess optimization, the response surface methodology (RSM) is the most suitable one for identifying the effect of individual independent variables and their interactions (Belwal et al., 2016; Myers et al., 2016). This statistical method allows the simultaneous variation of all parameters (pH, temperature, etc.) to measure the impact on the dependent variable (ethanol production) and to determine the impact of the interaction between the parameters. This statistical method is also suitable because it enables the efficient determination of the optimal conditions for a multivariable system (Montgomery and Runger, 2003).

3.2.1. The Regression Model

Table 3 presents the real (actual) and the predicted ethanol production obtained from each run. It shows an excellent agreement between the real values of ethanol production and predicted ones.

Indeed, the small residual value (i.e. the difference between real and predicted ethanol production values), ranging between 0 and 0.27 (g L^{-1}), proves the reliability of the obtained results.

Table 3. Real and predicted ethanol (g L^{-1}) produced by *K. marxianus* YMEK23

Run	*Ethanol Production (g L^{-1})*		
	Real	*Predicted*	*Residual*
1	38.62	38.41	0.208
2	38.62	38.83	-0.211
3	33.73	33.51	0.211
4	34.25	34.45	-0.208
5	36.43	36.70	-0.271
6	36.43	36.57	-0.148
7	37.53	37.38	0.148
8	37.53	37.25	0.271
9	40.82	40.75	0.062
10	36.43	36.37	0.06
11	40.82	40.88	-0.06
12	35.94	36.00	-0.062
13	38.62	38.62	0.00
14	38.62	38.62	0.00
15	38.62	38.62	0.00

3.2.2. Statistical model and its validation

The Analysis of Variance (ANOVA), shown in Table 4, indicates that the response surface model developed for ethanol production is statistically significant, with a p-value inferior to 0.0001.

Table 4. Analysis of Variance (ANOVA)

Source of variance	*df*	*Sum of squares*	*Mean square*	*Report F*	*p-value*
Model	9	58.621	6.5069	84.98	<0.0001
Error	5	0.38283	0.0766		
R^2	99.35%				
R^2aj	98.18%				

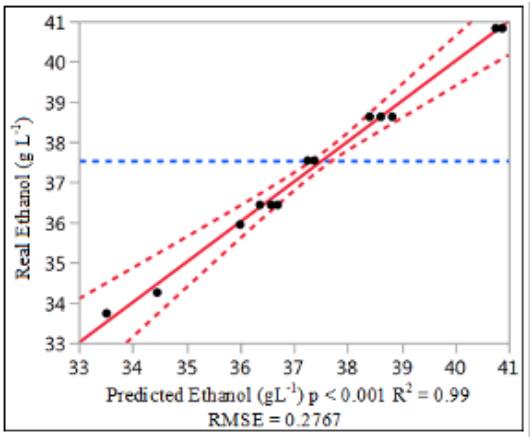

Fig. 1. Representation of real values as a function of predicted values

The fit of the model was also checked by the coefficient of determination R^2 which equals 0.9935, indicating that 99.35 % of the variability in the response (i.e. dependent variable) is explained by the independent variables of the model. Montgomery (2019) explains that the model is statistically significant and well fitted if the coefficient of determination is greater than 0.8. Besides, Fig. 1 shows a strong correlation between the experimental results and the theoretical values predicted by the statistical model as all data points are scattered around a straight line.

The regression analysis results of the dependent variable Y (i.e. ethanol production in g L^{-1}) as a function of the linear terms b_1 (i.e. temperature in °C), b_2 (i.e. pH), and b_3 b_3 (i.e. nitrogen supplementation in g L^{-1}) and their interaction as well as the quadratic terms b_1^2, b_2^2, and b_3^2 shown in Table 5 indicate that the terms b_1, b_2, b_1^2, and b_2^2 are statistically significant at 95% confidence level (i.e. p <0.05) while b_3, b_3^2 and the interaction terms are not statistically significant at the same confidence level. As a result, the statistically insignificant terms are dropped from the model using ANOVA backward elimination procedure. The final equation for the ethanol production model as a function of the coded factors is given as Eq. (2):

$$Y = 38.62 - 2.32X_1 + 0.34X_2 - 0.40X_1^2 - 1.92X_2^2 + \varepsilon \quad (2)$$

Table 5. Estimated coefficients of polynomial equation for investigated dependent variables using coded values: X_1-Temperature (°C), X_2-pH, X_3-Dose of Nitrogen Supplementation (g L^{-1})

Term	*Code*	*Coeffi-cient*	*Standard Deviation (SD)*	*Report t*	*p-value*
Constant	b_0	38.62	0.159755	241.75	<0.0001*
X_1	b_1	-2.32	0.09783	-23.68	<0.0001*
X_2	b_2	0.34	0.09783	3.48	0.0177*
X_3	b_3	-0.06	0.09783	-0.63	0.5587
X_1*X_2	b_{12}	0.13	0.138352	0.94	0.3905
X_1*X_3	b_{13}	-0.12	0.138352	-0.89	0.4165
X_2*X_3	b_{23}	0.00	0.138352	0.00	1.0000
X_1*X_1	b_1^2	-0.40	0.144001	-2.75	0.0402*
X_2*X_2	b_2^2	-1.92	0.144001	-13.32	<0.0001*
X_3*X_3	b_3^2	0.28	0.144001	1.94	0.1107

Fig. 2 shows the effect of the independent variables on ethanol production ranked in order of importance. It is noticed that temperature and pH*pH are the two main factors influencing ethanol production. Coefficients analysis demonstrates that ethanol production is more influenced by temperature than it is influenced by the square of the pH. Similar results were found for the optimization of cactus pear fruit fermentation for wine production (Tsegay et al., 2018). The negative sign of temperature and pH*pH coefficients indicates that ethanol production is inversely proportional to these two parameters. In other words, ethanol production decreases as temperature and pH*pH increase.

Fig. 3 shows the interaction between temperature and pH in influencing ethanol concentration. It is noticed that the maximum quantity of this metabolite is obtained at low temperatures (i.e.

36 and 41°C) and intermediate pH (4 and 6). Yan et al. (2012) reported similar results.

3.2.3. Desirability Research

Desirability research by JMP software makes it possible to find the optimal setting of the parameters that lead to maximum ethanol production. The optimization of the response is achieved by considering the least desirable value as zero and the most desirable value as one (Montgomery, 2019). Fig. 4 shows that an ethanol production of 41 g L^{-1} from prickly pear juice is achievable, with 100% desirability, at 37°C incubation temperature and a pH of 5.

3.3. Kinetics of ethanol production

The kinetics of batch fermentation is carried out with optimized parameters which are a temperature of 37°C and a pH of 5 with 6 10^8 yeast cells/mL inoculation of the culture medium.

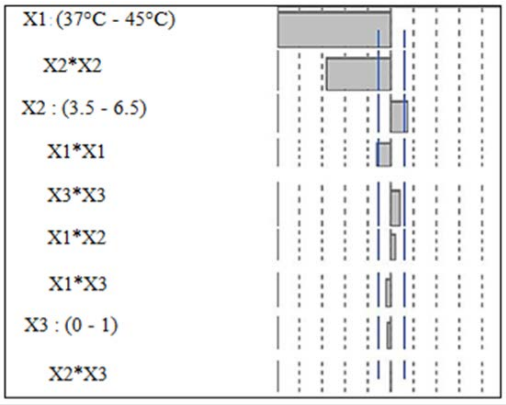

Fig. 2. Ranking of factors influencing ethanol production ordered by importance

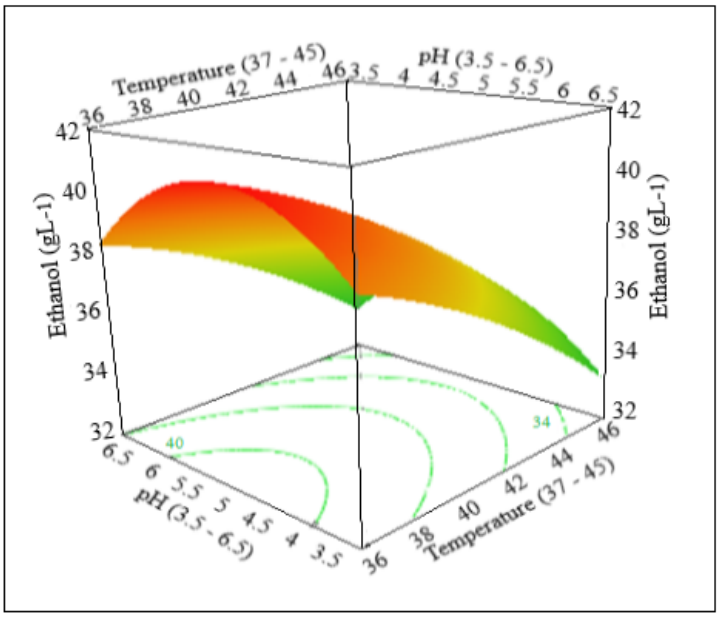

Fig. 3. Three-dimensional plot, showing the effect of pH and temperature on the ethanol concentration after fermentation (Nitrogen supplementation was fixed at 0 coded level)

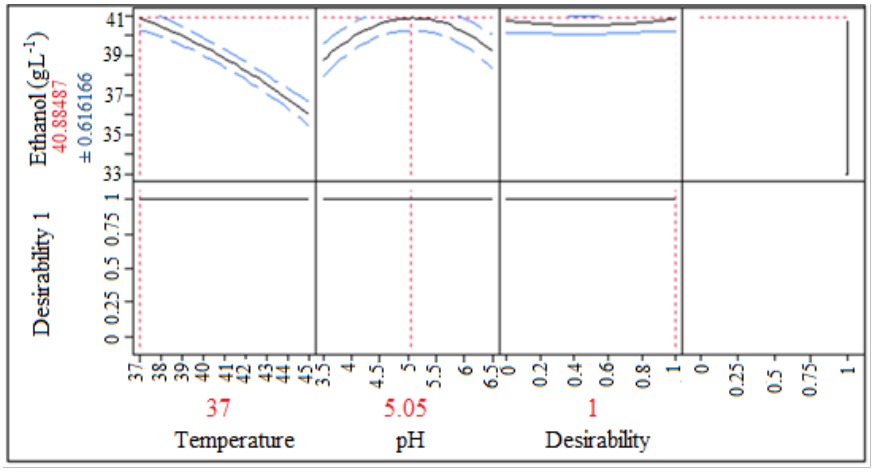

Fig. 4. Forecasting profile of optimal ethanol production conditions

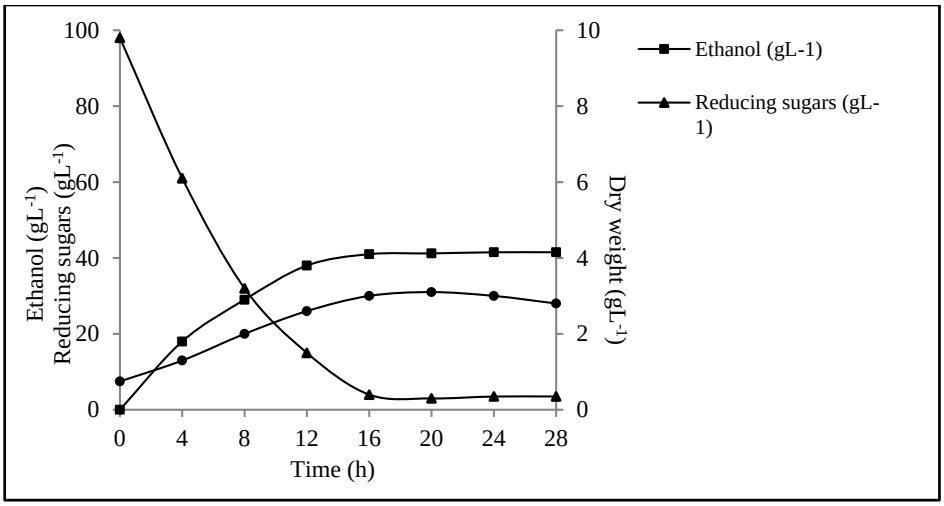

Fig. 5. Kinetic of batch fermentation production of ethanol from prickly pear fruits juice with *K. marxianus* YMEK23 at 37°C, pH 5. Aliquots of 10 mL were aseptically taken each 4h to quantify: (■) dry weight, (●) ethanol and (▲) reducing sugars

Fig. 5 illustrates ethanol and cell biomass production as well as substrate consumption as a function of time. The shape of the graphs in Fig. 5 shows a very active fermentative metabolism of prickly pear juice by *K. marxianus* YMEK23, reflected by the absence of the latent phase, early and exponential production of ethanol as well as a rapid consumption of sugars. Accordingly, the maximum quantity of ethanol (41 g L^{-1}) is reached after only 16 h of fermentation, with a yield of 0.43 g g^{-1} and a productivity of 2.56 g L^{-1} h^{-1}. On the one hand, the high metabolic activity can be attributed to prickly pear juice's richness in simple and easily fermentable sugars such as glucose and fructose. On the other hand, it can be attributed to the adaptation of *K. marxianus* YMEK23 to prickly pear juice as a culture substrate for ethanol production.

In this study, *Saccharomyces cerevisiae* YMES2 was used as a control and gave a similar optimization profile to that of *K. marxianus* up to a temperature of 41°C (data not shown). Above this temperature, the amount of ethanol produced by *S. cerevisiae* is much lower than *K. marxianus* which continues to produce ethanol at 45°C and pH of 5, despite a decrease in productivity varying between 10% and 33% depending on the duration of the fermentation cycle. This finding validates our choice for using the thermoresistant ethanologenic *K. marxianus* yeast for this process.

This is the first study that addresses the feasibility of *K. marxianus* fermentation of cactus prickly pear fruit for bioethanol production instead of *Opuntia ficus-indica* cladodes. Dried cladodes of *Opuntia ficus-indica* were used to produce up to 19.5 g L^{-1} second-generation bioethanol using *K. marxianus* in 40 h fermentation cycles at 40°C (Kuloyo et al., 2014). Dominguez et al. (2018) reported an ethanol production of 11.7 g L^{-1} after hydrolysis of cellulose in cladodes by *Acinetobacter pittii* bacteria and semi-simultaneous saccharification and fermentation (SSSF) by *K. marxianus*. Such a longer and multistep fermentation process produced a lower yield than the process described in the current study.

4. Conclusions

The present work demonstrated that *Opuntia ficus-indica* juice is a suitable feedstock for ethanol production due to its high sugar content (98 g L^{-1}). Response Surface Methodology (RSM) proved to be useful in optimizing the conditions for maximum ethanol production. As demonstrated, the maximum ethanol concentration of 41 g L^{-1} was obtained after a short fermentation cycle of 16 hours at 37°C and pH 5 without supplementing a nitrogen source to the medium.

K. marxianus YMEK23 used in the implementation of the fermentations showed a significant adaptation to prickly pear juice as a culture substrate, translated by high productivity of 2.56 g $L^{-1}h^{-1}$. Given the Crabtree-negative metabolism of *K. marxianus*, the adoption of feed batch fermentation with controlled aeration will undoubtedly lead to the improvement of all process parameters. Fermentation at temperatures between 37 and 45°C is also feasible using this yeast species. The optimization of the fermentation process at high temperature represents a considerable advantage for countries with a warm climate like Morocco. It also enables the reduction of the energy needed to cool the fermenters and eliminates the risk of contamination by undesirable micro-organisms.

Given its ability to withstand dry conditions and the fact that its cultivation does not require fertile land or irrigation, prickly pear cactus is found worldwide. Furthermore, this plant has high carbohydrate content and does not impinge on food availability.

These specificities represent strong arguments toward its exploitation in biofuel production. It represents an attractive and economical biomass alternative to conventional substrates currently used in the agro-fuels industry.

To optimize further the ethanol production from prickly pear we are working on the fermentation of the rest of the fruit which is composed of fiber and the fruit rind as a feedstock for the production of second-generation bioethanol using a simultaneous saccharification and fermentation.

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