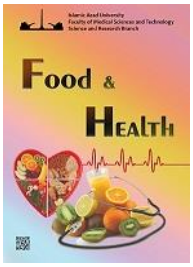

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Evaluation and statistical optimization of process variables for xylitol production by *Candida kefyr*

Fatemeh Hedayati Rad [1], Anousheh Sharifan [1*]

[1] Department of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran

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ABSTRACT

Xylitol is a pentahydroxypentane, which has high functional properties in food and drug industries. Biotechnology method has been investigated to overcome the drawbacks of xylitol chemical manufacturing. The effect of temperature, agitation speed, pH, and xylose concentration variables on xylitol production by *Candida kefyr* (ATCC 38296) was studied. The qualitative and quantitative of produced xylitol were evaluated by thin layer chromatography and high-performance liquid chromatography, respectively. The process optimization was done using response surface methodology (RSM) combine statistical experimental factorial designs. The 3D plots of xylitol production were proven to have the dependency and interaction between variables. The predicted results were close to the experimental results, which showed that the quadratic equation obtained from RSM was efficient. The dependency of xylitol yield on the interaction between variables was proven. Optimal levels of process variable were temperature of 28.15ºC, agitation speed of 132.34 rpm, pH value of 6.05, and xylose concentration of 58.90 g/L. On the third day of fermentation, the maximum yield was achieved (15.88 g/L). This is the first report of producing xylitol by *Candida kefyr* which has strong potential for bioconversion of xylose to xylitol with maximum yield and properties. This natural polyalcohol has desirable physicochemical and functional properties either in foods or in medicines.

1. Introduction

Xylitol is a natural five-carbon sugar polyalcohol which can be applied in pharmaceuticals and food industries due to its advantages such as appropriate sweetening power, physicochemical, anticarcinogenic and antioxidant properties (1, 2). Although this sugar and sucrose have an equal level of sweetness, it raises blood sugar level less than sucrose and insulin is not needed to regulate its metabolism (3, 4). Regarding to this reason, xylitol is used as a sugar substitute for diabetics in the pharmaceutical industry (5). This sugar can be added to toothpaste and chewing gums for promoting oral health and to reduce the dental cavities on root surfaces (2, 6). This low-calorie sugar can be a desirable alternative to sucrose in the dietary food manufacturers such as chocolate, mints, drinks, sweets and bakery products (2, 5, 6). Presently in industrial scale, a chemical route is used for xylitol manufacture by catalytic (nickel, ruthenium, rhodium, and palladium) hydrogenation of D-xylose (1, 4, 7). Chemical reduction of xylose involves several disadvantages such as high cost condition (aeration, high temperature, and pressure), expensive purification to separate main product from the other by-products such as sucrose or sorbitol and ethanol (2, 3). In addition, for reducing purification cost, D-xylose is purified by operation units which consist of ion exchange, chromatographic separation and discoloration (7). In contrast, biotechnological production is easy and environmentally-friendly, while having no toxic residue of catalyst (2). There are Several microorganisms to be mentioned including bacteria such as *Escherichia coli* (8), *Corynebacterium* sp. and *Enterobacter* sp. (9), filamentous fungi such as *Petromyces albertensis* (10) and many species of yeast such as *Pichia guilliermondii* (11), *Debaryomyces hansenii* (12), *Pachysolen tannophilus* (13), *Saccharomyces cerevisiae* (14) and *Rhodotorula mucilaginosa* (15), in addition some *Candida* species such as *Candida tropicalis* (16), *Candida* sp. ZU04

*Corresponding author: Department of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
E-mail address: a_sharifan2000@yahoo.com (Anousheh Sharifan)

(17), *Candida guillermondii* (18) and *Candida biodinii* (19). In previous studies, the possibility of xylitol production from xylose by *Candida kefyr* has not been studied. It is essential to optimize process condition, such as temperature, pH, oxygen concentration, aeration rate and concentration of monosaccharide and vitamins to increase the possibility and feasibility of xylitol industrial production (4, 5). Evaluation and determination of optimal process condition require statistical technique utilization; moreover, classic methods need a large number of experiments and are time consuming (1). To overcome such restrictions and study a large number of variables, response surface methodology (RSM) which combines statistical experimental factorial designs has been suggested (1, 3). This technique is vastly used for the optimization of biotechnological process (3). This work aimed to evaluate the possibility of xylitol production from xylose by *Candida kefyr* and the effect of temperature, agitation speed, pH, and xylose concentration on xylitol yield. These variable optimizations were quantified by RSM. The produced xylitol was analyzed quantitatively and qualitatively by thin layer chromatography and colorimetric methods, respectively.

2. Materials and methods

2.1. Materials

The *Candida kefyr* (ATCC 38296) lyophilized stock culture was collected from the Tehran University collections (Tehran, Iran). This stock culture was maintained on YPD (yeast extract peptone dextrose agar) at 4 ºC and sub-culture to preserve viability in every 1-2 months. Analytical grade chemicals contain D-xylose, ammonium sulfate, magnesium sulfate, potassium dihydrogen phosphate, yeast extract, peptone, propanol, butanol, potassium permanganate, sodium hydroxide, and sulfuric acid were purchased from the Merck Company (Darmstadt, Germany).

2.2. *Methods*

Fermentation was done in 100 ml Erlenmeyer flasks containing xylitol production medium (40 g/L xylose, 5 g/L ammonium sulfate, 2 g/L potassium dihydrogen phosphate, 0.5 g/L magnesium sulfate, 5 g/L yeast extract and 1 g/L peptone). Those mediums were sterilized at 121˚C for 15 mins and the cooling flasks were inoculated by activated *Candida kefyr*. The fermentation was carried out at 30˚C with 180 rpm. After 48 h, 5% of inoculation was added to fresh xylitol production medium and the effects of different parameters including xylose concentrations of 20, 40 and 60 g/L, temperatures of 25, 30 and 35˚C, pH values of 3.5, 5.5 and 7.5 and agitation speeds of 130, 180 and 230 rpm on xylitol production were studied for 5 days.

2.3. Qualitative evaluation of xylitol production using thin layer chromatography (TLC)

The produced xylitol was qualitatively analyzed by TLC technique. Amount of l µL of each medium containing xylitol and standard solution of xylitol (as a blank) had been applied on the silica plate of TLC (60F254) and was placed on TLC chamber for sufficient time to development of spots. The chamber contained a mobile phase, which consisted of solvent mixture including propanol-butanol-water with a ratio of 7:2:1. The plate was sprayed with potassium permanganate and sodium hydroxide to make the appearance of blemishes and the xylitol production was analyzed.

2.4. Quantitative evaluation of xylitol, residual xylose, and biomass

The amount of xylitol was determined by high-performance liquid chromatography (Shimadzu Prominence HPLC). In addition, the concentration of residual xylose and biomass was measured with this method. Refractive index detector and Nucleosil 100-5 NH_2 amine column (Knauer, Germany) were used for this method. The flow rate of mobile phase (H_2SO_4 0.01 M) and column oven temperature were maintained at 0.6 mL/min and 65˚C. Each sample was diluted in deionized water and was filtered. Then, 20 µL of the prepared sample was injected into the device. To quantify the unknown samples, standards were prepared and injected. The surfaces under the curve were calculated, and the standard curve was prepared.

2.5. Statistic optimization of xylitol production

Design-Expert software (version 8.0.7.1, Stat-Ease, Inc., USA) was used in the response surface methodology (RSM) with a 29 full factorial experimental design for process optimization. Among different classes of designs of RSM, the central composite design (CCD) was used for the optimization of the process variables. The relationship between the process variables and response variable with a minimum number of experiments were determined by the CCD and contour plots.

3. Results

The CCD of RSM was used for determination of the levels of fermentation variables which included temperature, pH, xylose concentration and agitation speed. The experimental matrix of the test consists of 29 full factorial design, the predicted and observed xylitol concentrations shown in Table 1. In addition, the second order regression equation calculates the predicted xylitol yield as a function of 4-process variables by Eq. 1.

(Equation 1) $Y = -65.22715 + (4.03867 \times T) - (0.018323 \times Ag) + (6.75404 \times pH) + (0.091386 \times Xy) - (0.068463 \times (T)^2) - (0.59840 \times (pH)$

where, Y (g/L), T (˚C), Ag (rpm) and Xy (g/L) are xylitol yield, temperature, agitation speed and xylose consecration, respectively. These values were close to those obtained from experimental xylitol yield.

Table 1. The effect of temperature (˚C), agitation speed (rpm), pH, and xylose concentration (g/L) on experimental and predicted xylitol yield.

Runs	Temperature (˚C)	Agitation speed (rpm)	pH	Xylose concentration (g/L)	Xylitol yield g/L	
					Experimental	Predicted
1	30	180	5.5	40	13.71	13.02
2	35	230	7.5	60	10.54	10.27
3	35	130	7.5	20	8.71	9.42
4	30	180	5.5	40	13.70	12.84
5	30	230	5.5	40	12.81	12.53
6	35	230	3.5	60	9.83	10.37
7	35	130	3.5	20	8.00	9.23
8	30	180	5.5	40	13.73	12.98
9	25	230	3.5	60	10.51	10.45
10	25	130	3.5	20	8.71	9.73
11	30	180	5.5	20	11.89	10.39
12	25	230	7.5	60	11.22	11.34
13	35	130	3.5	60	11.67	13.55
14	30	180	7.5	40	11.66	10.83
15	30	180	5.5	40	13.73	11.53
16	30	180	5.5	40	13.73	13.71
17	30	180	5.5	40	13.70	13.52
18	30	130	5.5	60	15.56	14.58
19	25	130	3.5	60	12.37	11.84
20	25	180	5.5	40	12.35	11.64
21	35	230	3.5	20	6.19	8.48
22	30	180	3.5	40	10.98	10.73
23	35	180	3.5	40	11.65	11.65
24	35	230	7.5	20	6.86	9.32
25	30	180	5.5	40	13.73	13.64
26	30	130	5.5	40	14.63	12.77
27	25	230	7.5	20	7.55	8.43
28	25	130	7.5	20	9.40	10.32
29	35	130	7.5	60	12.37	11.69

Fig. 1 (A, B, C, D, E, and F) shows the 3D curves of produced xylitol against any two independent variables while keeping the other variables at their central level. The Fig. 1A shows the dependency of xylitol yield on agitation and temperature. Increasing temperature up to 30˚C with 130 rpm agitation speed leaded to increase the fermentation yield but a further increase of these variables resulted in a decrease in the mentioned yield. The lowest production yield was observed at 35˚C at 230 rpm. As observed in Fig. 1B, The Parallel increase of temperature from 25˚C to 35˚C and pH from 3.5 to 5.5 resulted in an increase the xylitol production; therefore, it decreased with a further increase of them. Fig. 1C shows the dependency of xylitol production on xylose concentration and temperature. At concentration of 60 g/L of xylose, xylitol production reached to the maximum amount at 30˚C but with further increase in temperature, production yield decreased. Fig. 1D illustrates the effects of the pH changes and agitation speed on xylitol production. In lower agitation speeds, changes of pH value from 3.5 to 5.5 led to higher xylitol production by *Candida kefyr*; in addition, the higher pH value reduced production yield. The minimum yield was observed at pH value of 7.5 and agitation speed of 230 rpm. The dependency of xylitol production on xylose concentration and agitation speed is displayed in Fig. 1E. The maximum production was observed with 130 rpm of agitation speed and 60 g/L of xylose consecration but further agitation speed led to a decrease in xylitol production. The increase in pH value increased xylitol production up to 5.5 and thereafter the yield decreased with the further increased in pH value (Fig. 1F). The produced xylitol was qualitatively analyzed by TLC technique. The silica plate of TLC that was spotted with produced xylitol and standard solution of xylitol was shown in Fig. 2. The Design-Expert software determined the optimal amount of process factors to produce the highest amount of xylitol. These optimal levels of process condition consist of the temperature of 28.15˚C, pH value of 6.05, agitation speed of 132.34 rpm and xylose concentration 58.90 g/L. According to these optimized factors, the xylitol yield, biomass, and xylose were studied during 5 days of fermentation (Fig. 3). By increasing the xylitol production, the amount of biomass increased while the xylose decreased. The maximum amount of xylitol and biomass were obtained on the third day; in addition, the minimum amount of residual sugar was seen at the end of fermentation time.

4. Discussion

The production of xylitol by microorganisms is related to the activity of D-xylose reductase and xylitol dehydrogenase (6, 20). These enzymes are responsible for the metabolism of xylose, thereby for the reduction of xylose to xylitol. This bioconversion is strongly affected by temperature and pH (20). The optimum temperature for the activity of these enzymes is about 25-30˚C (6). The catalytic site of D-xylose reductase consists of tetrad of amino acids (Tyr, His, Asp, and Lys) damaged at high temperature (9). As our results showed, higher temperature led to a decrease in the production yield. Moreover, these findings were consistent with literature data

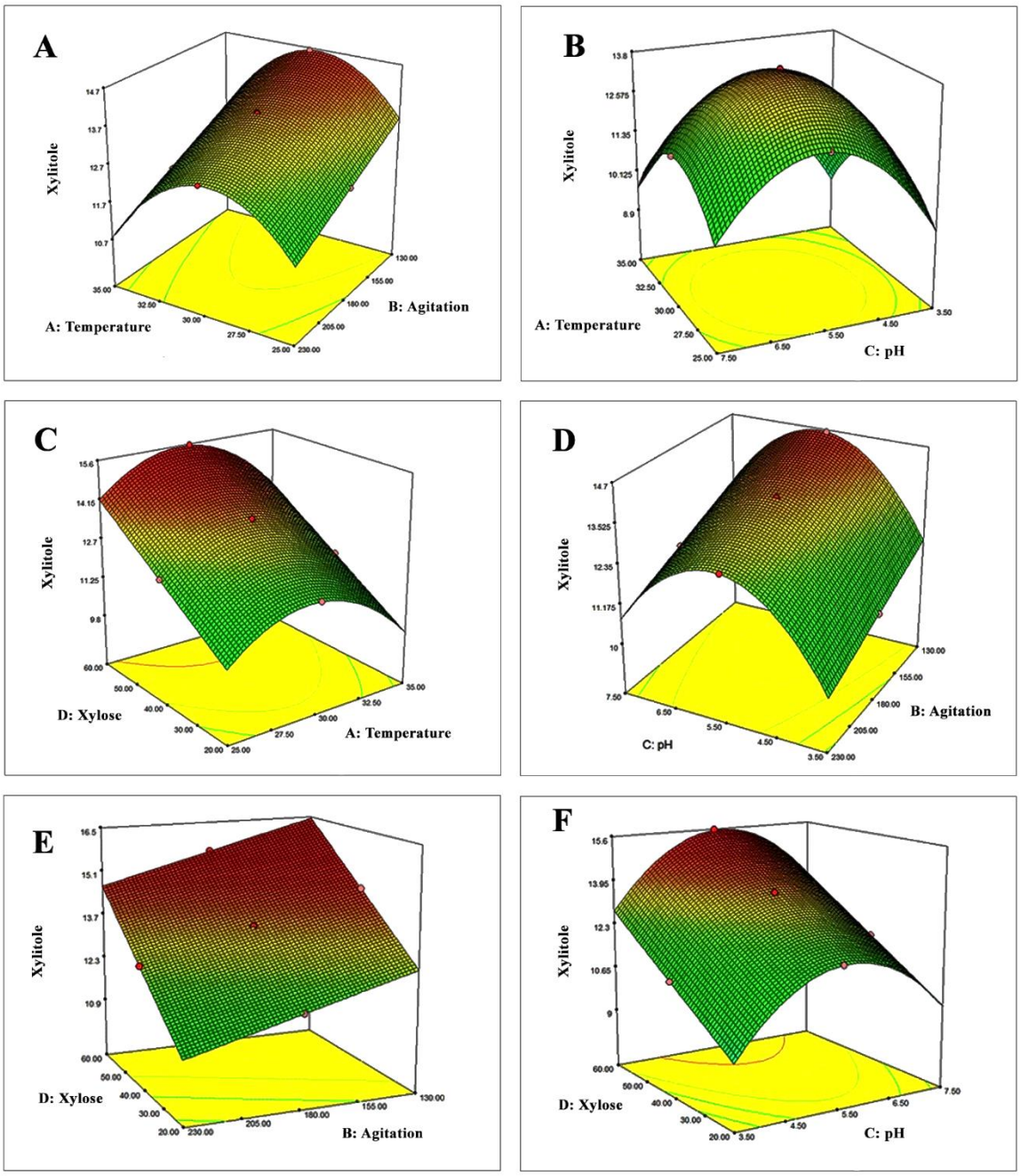

Fig. 1. The effect of temperature and agitation speed (A), temperature and pH (B), temperature and pH (C), pH and agitation speed (D), agitation speed and xylose concentration (E) and pH and xylose concentration (F) on Xylitol production.

(3, 21). The pH value of the yeast enzymes is a board range from 4 to 7 (4, 7). The lowest amount of xylitol yield was observed at 35˚C and pH value of 7.5. Ramesh et al. (3) Investigated, the interaction effects of pH, temperature, substrate concentration and agitation speed on xylitol production by RSM. They concluded that the increase in pH value (up to 7) and temperature (up to 31˚C) increased xylitol production yield. Interestingly, *Candida tropicalis* can be cultivated in very acidic conditions (pH=2.5) and provides a maximum xylitol yield (21). Another focal factor, which influenced xylitol yield, was xylose concentration (5, 21). Xylose is the most important substrate used by D-xylose reductase on the xylose fermentation (15). The minimum and the maximum amount of xylitol were obtained at concentrations of 20 g/L and 60 g/L of xylose, respectively. In Enshaeieh et al. (2) study showed that the best sugar concentration, in which 49/28 g/L of xylitol was achieved, was 140 g/L. Bura et al. (6) and Enshaeieh et al. (2) reported that the increase in the initial concentration of xylose was due to the higher yield of fermentation by *Rhodotorula mucilaginosa*. In contrast, due to substrate inhibition and/or osmotic pressure, the further increase in xylose concentration to 160 g/L in

Candida tropicalis fermentation led to a remarkable decrease in the production yield (5). Agitation of the fermentation medium provides oxygen for yeasts which enhance their growth and activity (21).

Fig. 2. TLC of xylitol production by *Candida kefyr*. Spot of produced xylitol (S) versus blank spot (B).

Aerated condition is desirable for xylitol production; moreover, the productivity yields were lower in low agitation speed. In this condition, longer fermentation time was required (4). The production yield is directly affected by the oxygen concentration because the generation rate of the necessary NADH or NADPH cofactors depends on it (17, 22). According to Mussatto et al. (23) study, at high agitation speed, the high volume of oxygen was entered into the fermentation medium, which led to obtaining a large number of cell masses. The consumption of the excess NADH, which was generated by extra oxygen volume, converted xylitol into xylulose and caused little accumulation of xylitol.

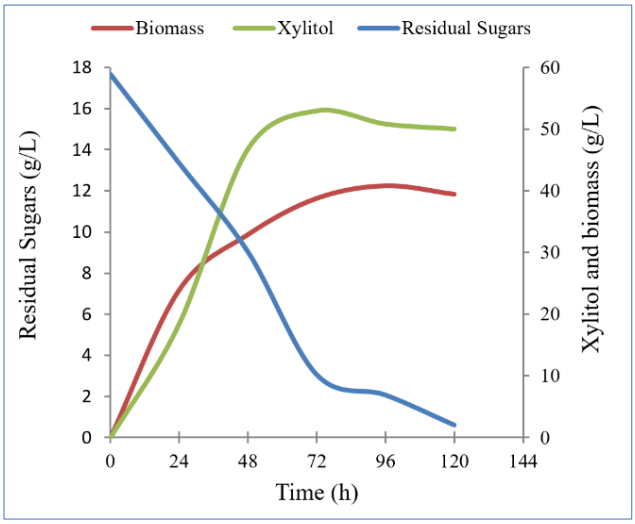

Fig. 3. The Xylitol synthetic plot at optimal fermentation conditions.

As shown in Fig. 1A, D and E, at low agitation speed, higher xylitol yield was achieved. Therefore, it can be concluded that although the high agitation rate increased the xylose consumption and biomass, it reduced the amount of xylitol accumulation. These results are in agreement with the findings of researchers that were found in the literature (3, 4, 23). The spot of produced xylitol placed versus blank spot qualitatively proved xylitol production by *Candida kefyr*. Enshaeieh et al. (2) evaluated produced xylitol by *Rhodotorula mucilaginosa* in the third day of fermentation with the TLC method and reported the same result. In the first day of the fermentation, the xylose concentration drastically reduced and vigorous biomass production was observed. After this phase, xylitol yield increased and there was less biomass formation. Similar results were reported in previous studies (1, 17). On the third day of fermentation as the exciting xylose in the medium was consumed, the maximum xylitol yield was observed and the production trend remained relatively constant until the last day. Undesirable fermentation conditions lead to consumption of xylose for cell growth and accumulation of biomass rather than for xylitol production (6).

5. Conclusion

With regard to the technology and economic aspects, the optimization of xylitol production by *Candida kefyr* is a pivotal point. Using an RSM approach, this study determined the effects of fermentation variables on xylitol yield which include temperature, pH, agitation speed and xylose concentration. All these factors had a marketable influence on the production yield. The findings of this optimization study are helpful for the production of xylitol with high yields.

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