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Response Surface Methodology Optimization Of Xylitol Production In Cells

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Abstract:
In order to enhance the production of xylitol through yeast fermentation, with the xylitol content in the yeast fermentation broth as the indicator, the response surface methodology was employed to optimize the fermentation conditions. Simultaneously, the basic physicochemical properties of intracellular amino acids in yeast cells were determined. Through single-factor experiments and response surface methodology, the optimal cultivation conditions for yeast fermentation to produce xylitol were determined as follows: initial glucose concentration of 30%, pH of 9.0, liquid volume of 25ml, and cultivation temperature of 28℃. In validated experiments under these conditions, the actual xylitol yield reached 47.53g/L, representing an 18.94% increase compared to the non-optimized conditions (initial glucose concentration of 30%, pH of 8, liquid volume of 25ml). Total nitrogen content determination and amino acid analysis of the cultured yeast cells revealed that the optimized conditions led to a higher proportion of amino acids such as arginine and glutamic acid, which enhance yeast cell vitality. Therefore, the use of response surface methodology effectively improved the yield of xylitol in yeast fermentation.
Background*: Xylitol, as a natural sweetener, is widely employed in various sectors such as food, pharmaceuticals, and cosmetics, offering advantages of low calorie content, low glycemic index, and no elevation of blood sugar. In order to meet the growing demand for low-sugar and low-calorie foods and the increasing focus on renewable bioresources, researchers have been actively seeking efficient biological methods for xylitol production. Traditional methods of xylitol production heavily depend on plant extraction, which is expensive and resource-limited. Therefore, the synthesis of xylitol through biological methods has become a highly regarded approach.Biological methods typically involve microorganisms, such as yeast, utilizing inexpensive carbon sources like glucose through the fermentation process to produce xylitol. This method not only boasts environmental and sustainable advantages but also expands the possibilities for biotechnological applications in the food and pharmaceutical industries. The ongoing in-depth exploration of this research field will contribute to the development of more efficient, economical, and environmentally friendly xylitol production processes, promoting the widespread application of xylitol.*
Materials and Methods*: Through orthogonal experimental design, we conducted response surface optimization of the fermentation conditions. Using SPSS, we planned experiments with various combinations of initial glucose concentrations, pH values, and liquid volumes. By simulating the fermentation model with software, we identified the optimal conditions for xylitol production and conducted validation experiments.*
Results*: After response surface optimization, the optimal fermentation conditions were determined as follows: initial glucose concentration of 30%, pH of 9.0, liquid volume of 25ml, and cultivation temperature of 28℃. Under these conditions, the xylitol yield reached 47.53g/L, an increase of 18.94% compared to the non-optimized conditions.*
Key Word*: Response surface optimization, xylitol, amino acids*

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I. Introduction

Xylitol, as a sugar substitute, finds wide applications in the food and pharmaceutical industries[1]. Currently, industrial production of xylitol predominantly relies on chemical hydrogenation methods[2], resulting in waste disposal issues and environmental pollution. Therefore, the development of a low-energy microbial approach for xylitol production, coupled with strategies to enhance xylitol yield through metabolic manipulation, has become a focus of contemporary research. Many studies utilize metabolic engineering and genetic engineering to manipulate metabolites and enzyme levels, thereby increasing the yield of the desired product[3]。 On the other hand, under similar fermentation conditions, the amino acid metabolism level of cells reflects their vitality, and higher cell vitality often correlates with increased product yield. In existing research reports, yeast species are often the natural producers of xylitol [4], with only a few strains from other genera, such as Escherichia coli and

Candida, capable of xylitol production through genetic modifications [5,6]. This preference for yeast species is attributed to the fact that microbial production of xylitol often occurs in high osmotic environments with high sugar concentrations (e.g., glucose, xylose). Under these conditions, yeast cell membranes exhibit a natural advantage [7], making yeast species generally more robust than other strains in high osmotic pressure environments [8]. Moreover, xylitol is known for its excellent properties, such as improving liver function, stabilizing insulin, and preventing dental caries. Consequently, it has widespread applications and considerable potential for high-value utilization in various fields, including biomedicine and the food industry. However, its low yield and associated pollution remain significant drawbacks, severely limiting its widespread industrial and food-related applications. Therefore, optimizing fermentation conditions and improving fermentation methods are active research directions.[9]

In this experiment, the response surface methodology was employed to optimize yeast fermentation conditions, with xylitol yield as the primary target. Simultaneously, protein content and amino acid analysis of the yeast were determined and analyzed.

II. Material And Methods

Strains: The yeast strain were obtained from the Comprehensive Laboratory of Food Science and Engineering at Shandong University of Technology.

Culture media: The seed culture medium consisted of 2% glucose, 1% peptone, 1% yeast extract, with a pH of 6.8. The solid culture medium comprised 2% glucose, 1% peptone, 1% yeast extract, 1.5% agar, with a pH of 6.8. The fermentation medium included 30% glucose, 1% yeast extract, 0.15% ammonium dihydrogen phosphate, 0.01% zinc sulfate, 0.05% magnesium sulfate, 0.05% potassium chloride, with a pH of 8. The solid selection medium contained 30% glucose, 1% peptone, 1% yeast extract, 1.5% agar.

Growth curve and biomass determination: Fermentation broth samples were collected at different fermentation times for quantitative analysis. The cells were obtained by centrifugation, washed with distilled water, and then stored at -80°C overnight. The samples were freeze-dried for 48 hours until a constant weight was achieved, and the cell dry weight was determined by weighing, representing the biomass of the cells. The growth curve of the cells was measured spectrophotometrically by recording the absorbance at 600nm.

Response surface optimization design: Based on the results of single-factor experiments, a response surface optimization experiment was conducted using the principles of the Box-Behnken design. Tea infusion volume fraction, sucrose mass fraction, cultivation temperature, and liquid volume were selected as independent variables, with the response value being the cellulose content of red tea fungus. The experimental design is presented in Table no 1.

Table no 1: Factor and Level Table for Central Composite Experimental Design

Factors	Levels		
	-1	0	1
A: Initial glucose concentration	25	30	35
B :pH	7.5	8.5	9.5
C: Liquid volume	20	25	30

Determination of residual carbon source, xylitol, and total nitrogen content: Carbon source and xylitol content were determined using high-performance liquid chromatography (HPLC). The chromatographic column used was Zorbax NH_2 (4.6×250 nm, 5μm), with a column temperature of 40℃. The detection was carried out using a refractive index detector (RID) at a temperature of 40℃. The mobile phase consisted of acetonitrile: water (8:2), and the detection time was set at 15 minutes.

The determination of total nitrogen content was conducted using the Kjeldahl method [10]. After digesting samples from different fermentation stages, the total nitrogen content of the samples was determined using a Kjeldahl nitrogen analyzer to quantify the nitrogen content in the samples.

Determination of amino acid content in yeast biomass: Using an automatic amino acid analyzer (LA8080, Japan) and ion exchange chromatography (Ostion Lg ANB column) to determine the amino acid content in yeast cell biomass. All samples underwent crushing and were hydrolyzed in 6M HCl at 110°C for 24 hours. After hydrolysis, the residual free HCl was dissolved in 2 mL of sample buffer (0.2 M, pH 2.2) and then injected into the amino acid analyzer. By following the method proposed by Zhao et al.[11], the determination of free amino acids was confirmed through the comparison of individual amino acid retention times and peak areas.

Statistical analysis

Using SPSS version 20 (SPSS Inc., Chicago, IL) software for statistical analysis of experimental data, Origin 2021 software for plotting single-factor experimental results, and Design Expert 12 for response surface experimental design and analysis.

III. Result and Discussion

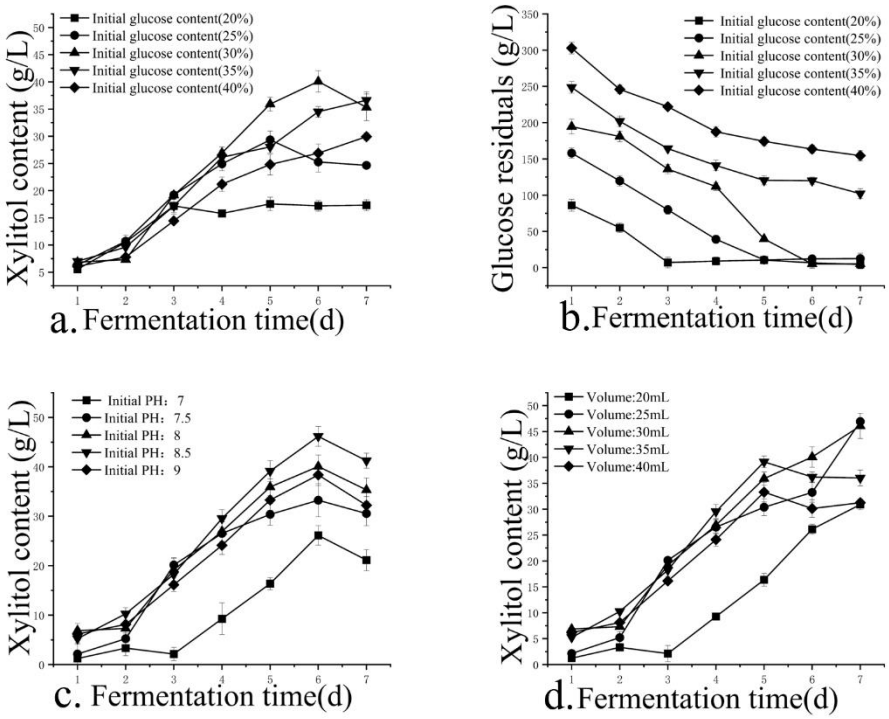
Single-factor experiment

According to Figure 1a and b, with the increase of initial glucose concentration in the fermentation broth, the consumption rate of glucose initially rises and then falls. Similarly, the production and conversion rate of xylitol exhibit an increasing trend followed by a decrease. When the initial glucose concentration is too low, the consumption of glucose is rapid, but the xylitol production is low with a lower conversion rate. However, at an initial glucose concentration of 30%, the consumption time of glucose is moderate, resulting in the maximum production of xylitol and the highest conversion rate, reaching 11.30%. Nevertheless, as the initial glucose concentration continues to increase, the utilization rate of glucose, xylitol production, and conversion rate all significantly decrease. This suggests that an excessively high glucose concentration may inhibit the cell respiration of the strain, thereby affecting the growth of yeast cells and leading to a decrease in xylitol production. This result is consistent with previous literature reports [12]. Considering both xylitol yield and conversion rate, we preliminarily choose an initial glucose concentration of 30%, which appears to be favorable for the conversion of xylitol by the yeast.

Similarly, in Figure 1c, as the initial fermentation pH increases, the xylitol production exhibits a trend of first increasing and then decreasing. Moreover, the production of xylitol gradually decreases with the increasing pH of the fermentation environment. In existing literature, it has been found that acidic conditions can inhibit the sugar transport mechanism on the cell membrane of the strain [13], thereby affecting the production of xylitol. Therefore, combining the experimental results with relevant literature analysis, we infer that the initial pH value of the fermentation conditions is also a crucial factor influencing xylitol production.

In Figure 1d, we observe that with the increase in liquid volume, there is a trend of initially increasing and then decreasing xylitol production, similar to the pH results. Considering that the yeast is a facultative anaerobe, it undergoes alcoholic fermentation under anaerobic conditions and sugar alcohol fermentation under aerobic conditions [14]. Therefore, the amount of liquid volume reflects whether the yeast undergoes aerobic or anaerobic fermentation. From the results, it can be observed that maintaining the liquid volume between 25-35mL ensures a higher production of xylitol.

Since all three single factors significantly influence xylitol production, we will proceed to optimize the fermentation conditions further using the response surface optimization method.



Figuer no 1: Shows the influence of single factors on xylitol production (a. Xylitol production during fermentation with different initial glucose concentrations; b. Residual glucose levels during fermentation with different initial glucose concentrations; c. Xylitol production during fermentation with different initial pH values; d. Xylitol production during fermentation with different initial liquid volumes)

Response surface optimization of fermentation conditions

Through single-factor experiments, we found that both initial glucose concentration and pH significantly affect xylitol production. Additionally, due to the positive correlation between the oxygen concentration of the yeast and xylitol production, the volume of liquid becomes particularly important. Therefore, in this experiment, with initial glucose concentration (A), pH (B), and liquid volume (C) as experimental factors, according to the principles of experimental design, xylitol production was taken as the evaluation index. We designed a response surface analysis with three factors and three levels, consisting of 17 experimental points. The zero-point experiment was repeated five times. The levels and coding of the experimental factors are detailed in Table 1, and the results of the response surface analysis are presented in Table 2. The variance analysis of the model and the significance test of the coefficients are summarized in Table 3.

From Table 3, it can be observed that the p-value for the lack-of-fit term is 0.2004, which is greater than 0.05, indicating no significant difference. Therefore, it can be concluded that the model fit is effective, and this model and equation can be used to analyze xylitol production.

The model's coefficient of determination (R_{adj}^2) obtained through software analysis is 99.49%, indicating the reliability of this experimental method. The model exhibits good data fitting and a small experimental error. From the results of the significance test of regression model coefficients, it can be observed that the influence of factor B is the most significant, the influence of factor A is significant, and the influence of factor C is not significant. The quadratic terms AA, BB, and CC in the equation are all highly significant. In the interaction terms, the influence of BC is highly significant, and the influences of AB and AC are significant, indicating a more pronounced interaction effect between pH (B) and liquid volume (C) on xylitol production. Therefore, the ranking of the three factors affecting xylitol production is as follows: initial pH > initial glucose concentration > liquid volume.

Table no 2 : Orthogonal experimental design plan and results

Experiment number	A	B	C	Xylitol yield（g/L）
1	-1	1	0	30.77
2	0	0	0	46.08
3	0	0	0	46.06
4	0	-1	1	21.52
5	0	0	0	47.29
6	0	1	-1	30.90
7	-1	-1	0	20.32
8	0	-1	-1	27.12
9	-1	0	-1	22.99
10	-1	0	1	25.69
11	1	1	0	30.75
12	1	0	-1	23.60
13	0	1	1	38.62
14	0	0	0	46.94
15	1	-1	0	15.92
16	1	0	1	21.91
17	0	0	0	45.94

Table no 3: Variance analysis of response surface regression coefficients

Source	SS	df	MS	F value	P value	Significance
Model	1912.52	9	212.50	349.38	<0.0001	**
A	7.18	1	7.18	11.80	0.0109	*
B	266.46	1	266.46	438.08	<0.0001	**
C	1.22	1	1.22	2.01	0.1991	
AB	4.80	1	4.80	7.90	0.0261	*
AC	4.82	1	4.82	7.93	0.0259	*
BC	44.36	1	44.36	72.93	<0.0001	**
A^2	825.85	1	825.85	1357.79	<0.0001	**
B^2	270.46	1	270.46	444.66	<0.0001	**
C^2	333.86	1	333.86	548.91	<0.0001	**
Res.	4.26	7	0.6082			
LOF	2.77	3	0.9230	2.48	0.2004	

Net error	1.49	4	0.3722			
Total	1916.78	16				

Ps: * is significant for the indicator（P<0.05）; ** is highly significant for the indicator (P<0.0001).

The interaction between pH (B) and liquid volume (C) on xylitol production is evident from the highest point and contour lines of the response surface, consistent with the results of the variance analysis. The optimal fermentation conditions obtained through response analysis are as follows: initial glucose concentration of 30.39%, pH of 8.97, and liquid volume of 25.10 ml. Under these conditions, the maximum xylitol production is 47.31 g/L, representing a 6.82% improvement compared to xylitol production in the unoptimized culture medium. To validate the reliability of the model simulation, experiments were conducted under actual conditions with an initial glucose concentration of 30%, pH of 9.0, and liquid volume of 25 ml. The experimental results showed a xylitol production of 47.53 g/L, which closely matched the theoretical predicted value, indicating the model's feasibility and effectiveness.

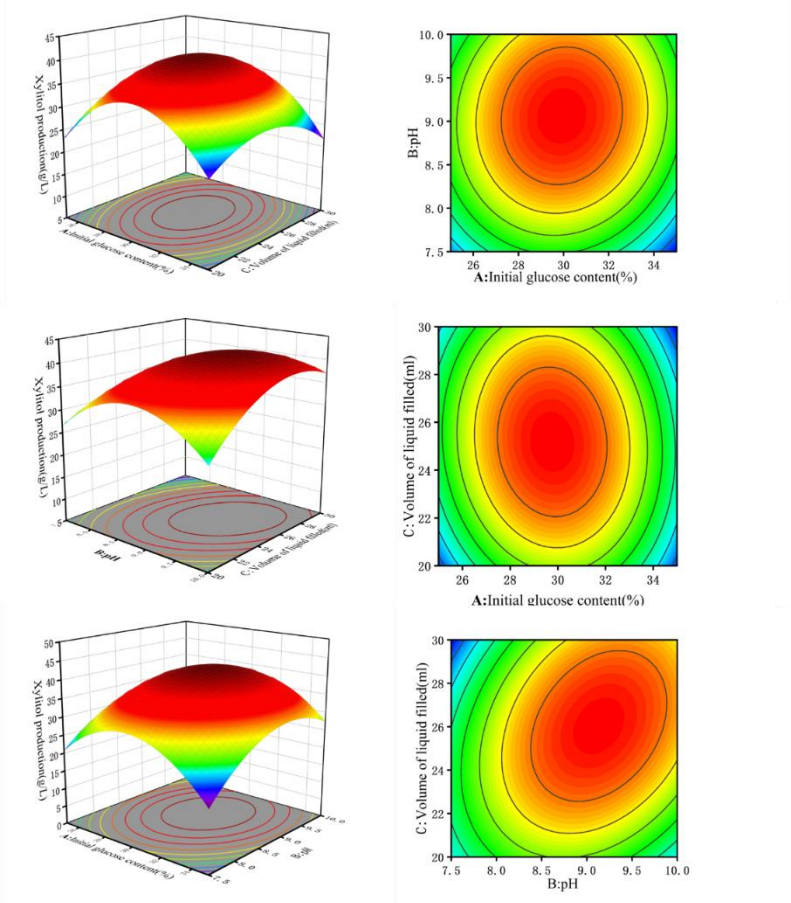


Figure no 2: Comparison of fermentation indicators of the yeast before and after response surface optimization

Fermentation properties determination

Due to the use of completely identical fermentation media by yeast, the key reason for the difference in strain xylitol production lies in the utilization rate of extracellular carbon sources and the generation rate of intracellular total nitrogen by the strains. Therefore, we measured the carbon source consumption rate and intracellular total nitrogen content during the yeast fermentation process (Figure no 3). Based on the change curve of xylitol production, a comprehensive analysis of the extracellular carbon source content and intracellular total nitrogen content during yeast fermentation was conducted.

In the figure, we can observe that strain rapidly consumes glucose within the first three days, and only a small amount of xylitol is produced. Combined with biomass measurements, we found that strain's primary fermentation task in the first four days is to increase its biomass by utilizing glucose. At this stage, only a small amount of xylitol is generated, indicating that strain directs very little carbon source towards xylitol metabolism in the first three days. Additionally, in our fermentation process [currently in progress], we have learned that glycerol content rapidly accumulates in the first four days. Interestingly, during the phase of rapid xylitol accumulation (days 3-5), both glucose and glycerol content decrease rapidly. In recent years, there have also been

reports of strains utilizing three-carbon glycerol as a co-substrate for fermentation, producing pentose xylitol[15]. Therefore, we believe that during the early stages of fermentation, a portion of glucose, moving towards the pentose phosphate pathway, accumulates triose phosphate glyceroneal leading to the byproduct glycerol. When biomass reaches its maximum, more 5-phosphoribulose ketone sugars start to accumulate, and simultaneously, glycerol is reversely decomposed, serving as a carbon source for xylitol production.

On the other hand, protein content is particularly important for yeast cell growth. Therefore, we measured the total protein content of the yeast cells. As shown in the figure, the intracellular total nitrogen (protein) content reached its peak within the first 4 days. From the results, it is evident that under optimized fermentation conditions, the strain exhibits a higher level of protein content, consistent with many existing research findings[16,17]. As precursors for protein synthesis, amino acids are essential for cell growth and reproduction. Additionally, amino acids participate in the biosynthesis of nucleotides and lipids, earning them the designation of 'fuel' for the energy needs of all living organisms[18].

Therefore, we conducted amino acid analysis on the fermentation broth of the strain on the fourth day, when the total nitrogen content reached its maximum (Figure no 3c). The average total of all amino acids in the optimized yeast was 251.317 ng/g. Exogenous amino acids determining the nutritional value of proteins accounted for approximately 49.5% of the total amino acid content[19]. Among them, the proportion of glutamic acid, phenylalanine, and methionine (Glu, Phe, Met) is relatively high, while the content of other amino acids is within the normal range. Suhajda et al. [20] found that the protein and individual amino acid content in yeast depend on the culture conditions and environment. Moreover, the protein content and the concentration of individual amino acids in yeast are mainly determined by genetic information. Therefore, under the same culture conditions and cultivation time, we conducted amino acid analysis on the strains and found that the optimized fermentation conditions played a decisive role in the significant increase of glutamic acid and methionine in yeast cells. On the other hand, research by Wu et al. [19] suggests that an increase in methionine and glutamic acid content can enhance the vitality of yeast cells and also affect the prolongation of yeast cell vitality. In other words, the increase in glutamic acid and methionine content in the strains after optimizing fermentation conditions may enhance and extend the vitality of yeast cells. Therefore, we believe that optimizing fermentation conditions have led to a higher metabolic level and cell vitality in the yeast, promoting the accumulation of xylitol.

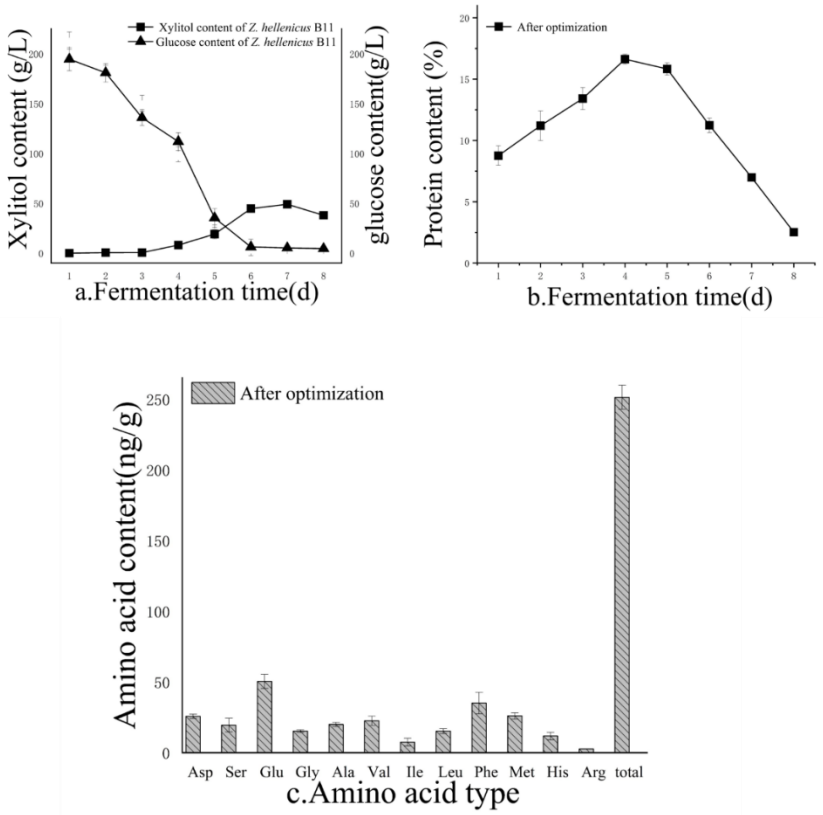


Figure no 3: Comparison of fermentation indicators of the strain before and after response surface optimization (a. Residual carbon source and xylitol production during fermentation of the optimized strain; b. Comparison of changes in intracellular total nitrogen content during strain fermentation; c. Intracellular amino acid content of the strain on the 4th day of fermentation)

IV. Conclusion

Cultivating strain for xylitol production using glucose as a carbon source, we determined the optimal fermentation conditions through single-factor experiments and response surface optimization. The best cultivation conditions were found to be: initial glucose concentration of 30%, pH of 9.0, liquid volume of 25ml, and cultivation temperature of 28℃. Experimental verification under these conditions yielded an actual xylitol production of 47.53g/L, representing an 18.94% increase compared to the unoptimized conditions (initial glucose concentration of 30%, pH of 8, liquid volume of 25ml, and cultivation temperature of 28℃). The response surface optimization effectively enhanced xylitol production.Tracking measurements during fermentation under optimized conditions revealed that the yeast exhibited increased cell vitality, with higher proportions of proteins, amino acids, and glutamic acid and valine. This enhancement in cellular vitality prolonged yeast cell activity, thereby promoting xylitol accumulation. These findings provide valuable guidance for selecting high-efficiency xylitol-producing strains and optimizing fermentation conditions in industrial production.

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