

L-Sorbose Production by *Gluconobacter oxydans* using Submerged Fermentation in a Bench Scale Fermenter

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Abstract

Background and objective: L-Sorbose, as a precursor of ascorbic acid, can be biologically produced using *Gluconobacter oxydans*. The aim of this study was to optimize production of L-Sorbose by controlling concentration of the substrates and starter cultures.

Material and methods: In this study, effects of three various fermentation parameters on the concentration of L-sorbose were assessed using fermenter (28°C, 1.4 vvm) and response surface methodology. These parameters included quantities of D-sorbitol (120-180 g l_{DW}⁻¹) (Deionized water) and yeast extract (6-18 g l_{DW}⁻¹) and inoculum/substrate ratios (5-10%).

Results and conclusion: Results showed that the fitted model with high values of R² (0.9594) and R²-adjusted (0.9228) could effectively predict the concentration of L-sorbose within the highlighted ranges for the variables. Furthermore, results demonstrated that the maximum concentration of L-sorbose was achieved at 42.26 g l_{DW}⁻¹ using D-sorbitol, yeast extract and inoculum/substrate ratio values of 153.42 g l_{DW}⁻¹, 12.64 g l_{DW}⁻¹ and 9.88%, respectively. These results have revealed appropriately of response surface methodology for the prediction of L-sorbose product quantity and optimization of the variables in this aerobic fermentation process.

Conflict of interest: The authors confirm that they have no conflict of interest.

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1. Introduction

Vitamin C or L-ascorbic acid is one of the soluble vitamins in the aqueous systems with high antioxidant activity, which makes it further helpful in pharmaceutical, cosmetic and food formulations [1,2]. Furthermore, this vitamin can be used as an additive to prolong shelf-life of the food products [3]. Vitamin C, as an important metabolite, includes several benefits for most of the living organisms such as plants and animals [4]. This essential nutrient includes various physiological properties, playing a key role in the most of human biosynthetic pathways. Unlike some plants and animals, humans cannot synthesis vitamin C due to the lack of L-gluconolacton oxidase. Therefore, the vitamin must be used in human dietary via foods or supplements [1]. Industrial production of vitamin

C through fermentation includes two major steps. In the first step, D-sorbitol is converted to L-sorbose by the activity of *Gluconobacter oxydans* [5]. The *G. oxydans* is an aerobic Gram-negative bacteria from acetic acid bacteria group, which can tolerate high concentrated solutions to oxidize alcohols and sugars [6,7]. In fact, the bacteria could successfully be used in novel biotechnology using chemical processes to produce numerous sugar derivatives and other valuable biological products [8]. In the second step, L-sorbose is bio-converted into 2-keto-L-gulonic acid using *Bacillus megaterium* and *Ktogulonigenium vulgare* [5, 9]. The chemical can then easily be converted to L-ascorbic acid.

While chemical oxidation of D-sorbitol is faster than microbial oxidation of this chemical and can produce L- and D-sorbitol sugars, incomplete microbial oxidation of D-sorbitol can selectively produce L-sorbose, which can increase yield of L-ascorbic acid, compared to that the chemical process can. Therefore, improvement of the production yield in the first step of microbial vitamin C production is important [10-12]. Studies have been carried out on the bio-conversion of D-sorbitol into L-sorbose through wild and genetically modified *G. oxydans* strains. Results have shown that the conversion yield is affected by multiple factors, including type and concentration of growth media, temperature, pH, dissolved oxygen concentration, inoculum/substrate ratio and type of fermenter [5,13,14]. Hence, the major objectives of the present study were 1) assessing effects of three fermentation parameters, including quantities of D-sorbitol (as carbon source) and yeast extract as nitrogen source) as well as inoculum/substrate ratio on production yield of the synthesized L-sorbose; and 2) optimizing fermentation conditions to achieve the highest L-sorbose concentration.

2. Materials and methods

2.1) Materials

D-sorbitol, L-sorbose, yeast extract, NaOH and H₂SO₄ were purchased from Merck, Germany. *G. oxydans* subsp. *suboxydans* (*Acetobacter suboxydans*) (PTCC 1051) was provided by the Persian Type Culture Collection (Iranian Research Organization for Science and Technology, Tehran, Iran).

2.2) Biomass preparation

To propagate the bacterial mass, culture media were prepared by adding 150 g of D-sorbitol and 12 g of yeast extract into 1 l of deionized water (D.W). Then, pH of the media was adjusted to 5.1 by adding NaOH (4% w w⁻¹) and H₂SO₄ (2.5% w w⁻¹) solutions. Culture media were sterilized using laboratory autoclave at 1.5 bar for 15 min at 121 °C. Then, 1 ml of D.W was added into the slant culture of *G. oxydans* in a tube and shaken well. Suspended bacteria were added into 50 ml of the prepared culture media at room temperature and transferred into a laboratory shaking incubator (Labcon 5082U, South Africa) at 150 rpm for 12 h at 30 °C.

2.3) Fermentation process

The L-sorbose was produced during fermentation process using 400-ml bioreactor (Figure 1). Briefly, 300 ml of the sterile culture media containing various quantities of D-sorbitol (120-180 g l_{DW}⁻¹) and yeast extract (6-18 g l_{DW}⁻¹) were prepared and various inoculum/substrate ratios (5-10%) were added into the culture media and mixed well. Mixtures were then transferred into a fermenter with 1.4

vvm aeration rate for 24 h at room temperature (~28 °C) (Table 1).



Figure 1. A 400-ml bioreactor, set at pH=5.1, T=28 °C and 1.4 vvm

Table 1. The fermentation conditions and their relative levels

Variable	Unit	Level		
		Low	Middle	High
X ₁ , quantity of D-sorbitol	g l _{DW} ⁻¹	120	150	180
X ₂ , quantity of yeast extract	g l _{DW} ⁻¹	6	12	18
X ₃ , inoculum to substrate ratio	%	5	7.5	10

2.4) Assessment of L-sorbose

After fermentation, L-sorbose concentration was assessed using high performance liquid chromatography Waters corporation, Milford, MA, USA) with a Sugar Pak Column (300 × 6.5 mm) (Waters corporation, Milford, MA, USA). The system was equipped with RI and UV detectors and D.W was used as mobile phase (eluent) with a flow rate of 0.3 ml min⁻¹ at 70 °C. Furthermore, L-sorbose solutions (40, 85 and 120 g l_{DW}⁻¹) were used as controls to demonstrate relationships between the voltage (provided by detectors of high performance liquid chromatography at 120, 260 and 360 mV, respectively) and concentration of L-sorbose and the peak area of the chemical (Figure 2).

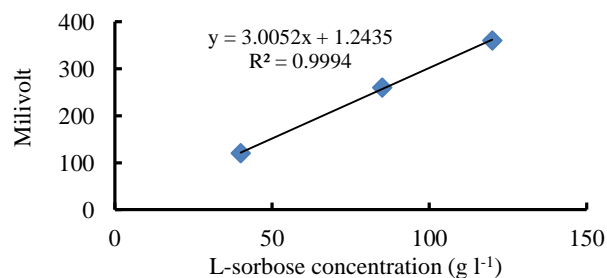


Figure 2. Results of Chromatograms of L-sorbose solutions as controls

2.5) Experimental design and data analysis

Central composite design was used to design experiments and response surface methodology (RSM) was used to correlate the independent fermentation process variables, including quantities of D-sorbitol (X1) and yeast extract (X2) and inoculum/substrate ratio (X3), to response variable of L-sorbose concentration (Y). Therefore, the center point of each variable range was calculated (Table 1) [15]. Indeed, RSM is one of the most efficient statistical methods, which maximally decreases the time of sampling and best discusses data from the experiments [16,17]. Of several standard designs used in experiments, central composite design can be a good choice since due to its high yields [18,19]. A general quadratic polynomial equation (Eq. 1) was used to indicate the main, quadratic and interaction effects of the selected fermentation variables on the concentration of produced L-sorbose:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

(Eq. 1)

Where, β_0 , β_i , β_{ii} and β_{ij} were constant, linear, quadratic and interaction coefficients, respectively. Coefficient of determination (R^2), adjusted coefficient of determination (R^2 -adj) and lack of fit (P-value) were used to assess appropriateness of the generated model based on the experimental data [20,21]. To better visualize effects of the independent variables on L-sorbose concentration, three-dimensional (3-D) surface plots and two-dimensional (2-D) contour plots were used. Moreover, numerical optimization was used to predict exact quantity of the optimum values for

the fermentation parameters [22,23]. To verify appropriateness and accuracy of the fitted model using RSM, additional 3-D experiments were carried out at optimum fermentation conditions and compression tests were carried out. Analysis of variance was set a significance level of $P \leq 0.05$. Design Expert Software (Trial v.7.0, Stat-Ease, Minneapolis, USA) was used for the experiment design and model generation, optimization and verification.

3. Results and discussion

3.1) Model generation

Based on the experimental design, 20 experiments, including six replicates for the center point, were carried out to assess values of the L-sorbose (Table 2). As shown in Table 2, concentration of the L-sorbose varied 25.3-45.4 g l_{DW}^{-1} . Based on the data of L-sorbose concentration and use of a second order polynomial equation, a general model was fitted for the prediction of L-sorbose concentration linked to D-sorbitol and yeast extract quantities and inoculum/substrate ratio. The P-values and coefficients of each term of the fitted model are shown in Table 3. The main terms of yeast extract quantity and inoculum/substrate ratio and quadratic effects of D-sorbitol and yeast extract quantities included significant effects on concentration of the produced L-sorbose ($P < 0.05$). The high values of R^2 (0.9594) and R^2 -adj (0.9228) demonstrated appropriateness of the generated model for the prediction of L-sorbose concentration within the highlighted ranges for the fermentation parameters.

Table 2. Matrix of the central composite design for the L-sorbose production by *Gluconobacter oxydans* PTCC 1051

Run	Amount of D-Sorbitol (g l_{DW}^{-1})	Amount of yeast extract (g l_{DW}^{-1})	Ratio of inoculum to substrate (%)	Amount of L-Sorbose (g l_{DW}^{-1})
1	120	18	5	26.3
2	150	12	7.5	41.1
3	150	12	10	45.4
4	120	6	5	24
5	150	12	5	36
6	150	6	7.5	31.3
7	150	18	7.5	33
8	150	12	7.5	41.2
9	180	12	7.5	35
10	120	12	7.5	38.2
11	150	12	7.5	41.1
12	180	6	10	32
13	180	18	5	31
14	180	6	5	25.3
15	120	6	10	28.2
16	150	12	7.5	41
17	150	12	7.5	39.8
18	180	18	10	33.4
19	120	18	10	33.1
20	150	12	7.5	40.3

Table 3. The *P*-values and regression coefficients for the generated model of *Gluconobacter oxydans* PTCC 1051

Parameter	P-value		Regression coefficient	
	Independent variable	<i>P</i> -value	β	Coefficient
Main	X ₁	0.2071	β_0 (constant)	-92.77
	X ₂	0.0107	β_1	1.07
	X ₃	0.0005	β_2	5.41
			β_3	3.34
Quadratic	X ₁ ²	0.01	β_{11}	-3.43×10^{-3}
	X ₂ ²	0.0001	β_{22}	-0.20
	X ₃ ²	0.4949	β_{33}	-0.11
			β_{12}	-6.94×10^{-5}
Interaction	X ₁ X ₂	0.9830	β_{13}	-3.16×10^{-3}
	X ₁ X ₃	0.6867	β_{23}	-0.01
	X ₂ X ₃	0.7180		
			R ² -adjusted	0.9228
			R ²	0.9594

1, D-sorbitol concentration (g l_{DW}⁻¹); 2, yeast extract concentration (g l_{DW}⁻¹); 3, inoculum/substrate ratio (%), X₁= D-sorbitol, X₂= yeast extract, X₃= inoculum/substrate ratio,

3.2) Effects of fermentation parameters on the concentration of produced L-sorbose

Effects of the selected fermentation parameters, including quantities of D-sorbitol and yeast extract and inoculum/substrate ratio (in highlighted ranges for each parameter), on L-sorbose concentration are listed in Figures 3-5. Figure 3a describes effects of D-sorbitol and yeast extract quantities on concentration of the produced L-sorbose. As seen in Figure 2a, by increasing quantity of the yeast extract with constant quantities of D-sorbitol, concentration of L-sorbose increased and decreased, respectively. A similar result was observed for changing quantity of D-sorbitol with constant quantities of yeast extract on concentration of the produced L-sorbose. Results revealed that interaction of the quantities of D-sorbitol and yeast extract insignificantly affected concentration of the produced L-sorbose ($P > 0.05$) (Table 3). As seen in Figure 3b, the maximum concentration of L-sorbose was achieved using D-sorbitol and yeast extract at nearly their center point quantities.

Figure 4a shows effects of D-sorbitol quantities and inoculum/substrate ratio on concentration of the produced L-sorbose. The figure clearly demonstrates that by increasing the inoculum/substrate ratio at constant quantities of D-sorbitol, the concentration of L-sorbose increased. A possible explanation is that by increasing the inoculum/substrate ratio, the population of *G. oxydans* increased in the fermenter. This subsequently increased the fermentation yield (quantity of the produced L-sorbose). This finding was similar to the finding by Shaghghi-Moghaddam et al. [24]. They reported that by increasing the inoculum concentration, concentration of the produced bioethanol by *Saccharomyces cerevisiae* increased. In contrast, by increasing the quantity of D-sorbitol at a constant inoculum/substrate ratio, concentration of the produced L-sorbose increased and decreased, respectively (Figure 4a).

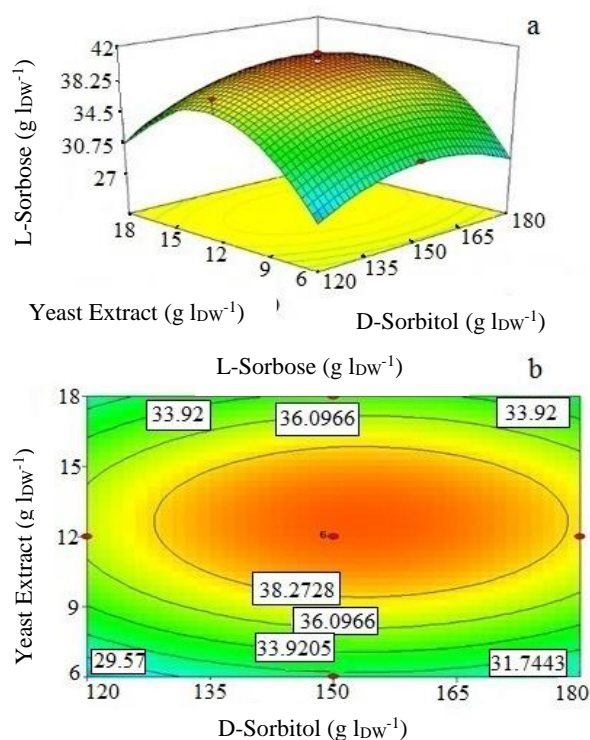


Figure 3. Surface plot (a) and contour plot (b) for the quantities of L-sorbose (g l_{DW}⁻¹) produced by *Gluconobacter oxydans* as a function of yeast extract and D-sorbitol concentration (g l_{DW}⁻¹)

It seems that by increasing the quantity of D-sorbitol, osmosis pressure of the fermentation culture media increased, which included inhibitory effects on *G. oxydans* growth as well as the bacterial specific action on the prepared substrate. Furthermore, this increase in osmosis pressure decreased the fermentation efficiency. This finding was similar to the finding by Edwards, who reported that high substrate concentrations might inhibit the bacterial growth and damage metabolism of the microorganisms [25].

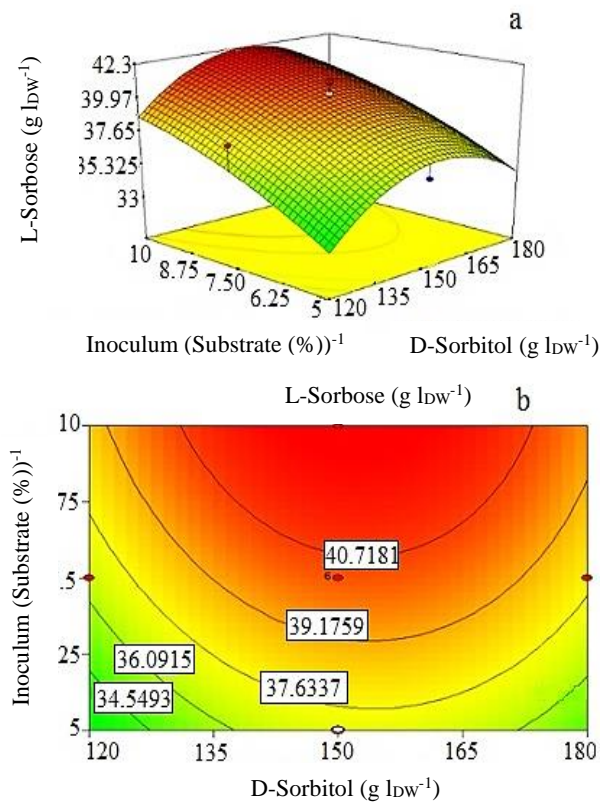


Figure 4. Surface plot (a) and contour plot (b) for the quantities of L-sorbose (g l_{DW}⁻¹) produced by *Gluconobacter oxydans* as a function of inoculum proportion (%) and D-sorbitol concentration (g l_{DW}⁻¹)

No curves in Figure 4a revealed insignificant effects of the inoculum/substrate ratio and quantity of D-sorbitol on concentration of the produced L-sorbose. This result was verified using the statistical data, which demonstrated that interaction between these two parameters was significant ($P=0.6867$) (Table 3). Figure 4b shows the optimum regions of the inoculum/substrate ratio and quantity of D-sorbitol. Based on the figure, the maximum concentration of L-sorbose was achieved at higher inoculum/substrate ratio and middle quantities of D-sorbitol. Figures 5a and 5b illustrate effects of the inoculum/substrate ratio and quantity of yeast extract on concentration of the produced L-sorbose; as reported by other researchers.

3.3) Optimization and validation procedures of the fermentation conditions

Numerical optimization, within the highlighted ranges for the three fermentation parameters, revealed that the maximum concentration of L-sorbose (42.26 g l_{DW}⁻¹) was achieved using D-sorbitol, yeast extract and inoculum/substrate ratio of 153.42 g l_{DW}⁻¹, 12.64 g l_{DW}⁻¹ and 9.88%, respectively. Using predicted values for the fermentation parameters, three additional experiments were carried out and concentration of the produced L-sorbose was

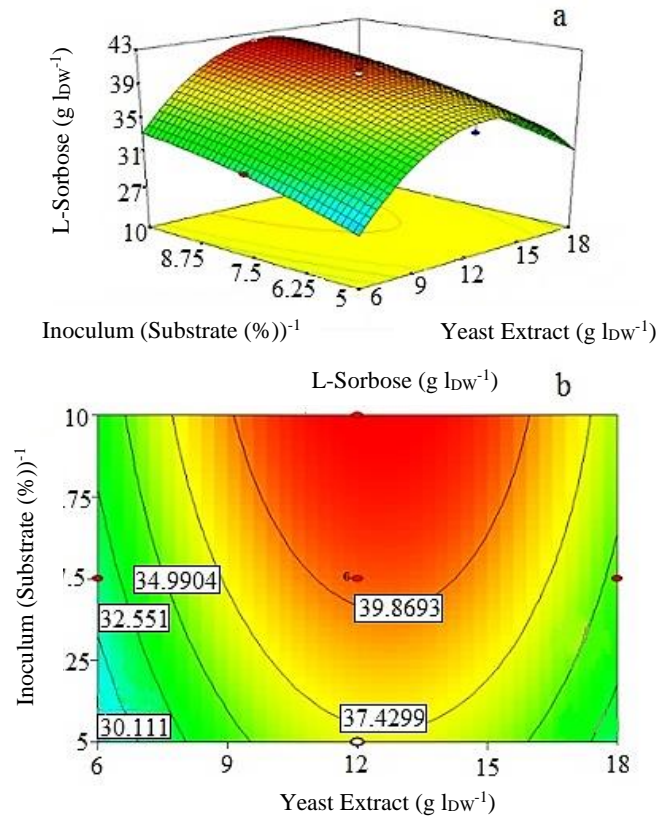


Figure 5. Surface plot (a) and contour plot (b) for the quantities of L-sorbose (g l_{DW}⁻¹) produced by *Gluconobacter oxydans* as a function of inoculum proportion (%) and yeast extract concentration (g l_{DW}⁻¹)

assessed as 45.4 g l_{DW}⁻¹ ± 4. Statistical analysis showed no significant differences between the predicted and experimental values for the concentration of produced L-sorbose at the highlighted fermentation conditions. Moreover, results demonstrated high accuracy and appropriateness of the fitted model based on the values of the experiments and central composite design. Figure 6 represents comparisons between the predicted and experimental values of the L-sorbose concentration.

4. Conclusion

In conclusion, high concentrations of L-sorbose were achieved in the present study using optimization of the fermentation conditions. However main and quadratic terms of the most variables showed significant effects on concentration of the produced L-sorbose ($P \leq 0.05$), their interactions showed no significant effects. It seems that optimization of other important fermentation parameters such as aeration rate, temperature and pH in this study has further increased the concentration of L-sorbose. Moreover, results have revealed the appropriateness of RSM in other biological systems.

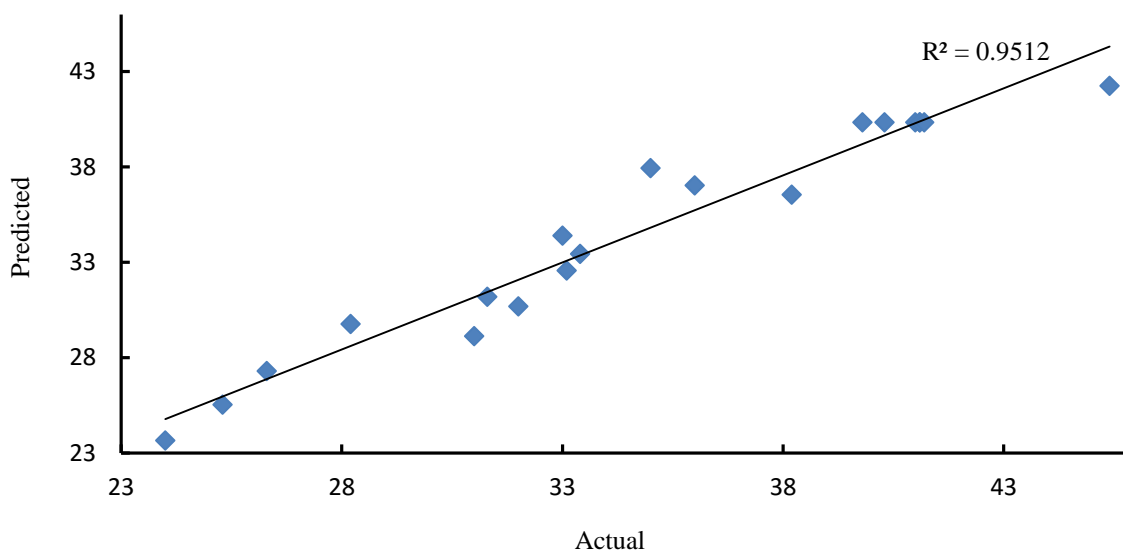


Figure 6. Predicted values against actual values for L-sorbose concentrations (g l_{DW}⁻¹)

5. Acknowledgements

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6. Conflict of interest

The authors declare no conflict of interest. No ethical approval was applicable in this study. No part of this study has previously been published or submitted for publication elsewhere.

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تولید ال - سوربوز توسط گلوکونوباکتر اکسیدانس با تخمیر غوطه ور در فرمانتور آزمایشگاهی

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چکیده

سابقه و هدف: ال - سوربوز، به عنوان پیش ساز برای تولید آسکوربیک اسید، توسط باکتری گلوکونوباکتر اکسیدانس به روش بیولوژیکی قابل تولید می باشد. هدف این مطالعه بهینه سازی تولید ال - سوربوز با کنترل غلظت رشدمایه ها و محیط های کشت آغازگر می باشد.

مواد و روش ها: در این مطالعه، اثرات ۳ متغیر فرایند تخمیر، شامل مقدار دی - سوربیتول (۱۲۰ - ۱۸۰ گرم بر لیتر آب دیونیزه)، مقدار عصاره مخمر (۶ - ۱۸ گرم بر لیتر آب دیونیزه) و نسبت تلقیح به محلول سوبسترا (۵ - ۱۰ درصد)، بر غلظت ال - سوربوز با استفاده از فرمانتور تحت شرایط دمای ۲۸°C و هوای ورودی ۱/۴ واحد حجمی به ازای واحد حجم محلول کلی بر دقیقه (vvm) و به روش سطح پاسخ مورد بررسی قرار گرفت.

یافته ها و نتیجه گیری: یافته ها نشان داد که مدل به دست آمده در روش سطح پاسخ، می تواند به طور مؤثری غلظت ال - سوربوز تولید شده در محدوده های مشخص متغیرها، با مقادیر بالای ضریب تعیین (۰/۹۵۹۴) و ضریب تعیین تعدیل شده (۰/۹۲۲۸) پیش بینی کند. علاوه بر این، نتایج نشان داد که حداکثر مقدار ال - سوربوز، ۴۲/۲۶ گرم بر لیتر آب دیونیزه با استفاده از ۱۵۳/۲ گرم بر لیتر آب دیونیزه دی - سوربیتول، ۱۲/۶۴ گرم بر لیتر آب دیونیزه عصاره مخمر و ۹/۸۸ درصد نسبت تلقیح به محلول سوبسترا به دست می آید. نتایج بیانگر این است که روش سطح پاسخ برای پیش بینی میزان تولید ال - سوربوز و بهینه سازی متغیرها در فرایند تخمیر هوای مناسب است.

تعارض منافع: نویسندگان تایید می نمایند که تضاد منافی ندارند.

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▪ تخمیر هوای
▪ آسکوربیک اسید
▪ گلوکونوباکتر اکسیدانس
▪ ال - سوربوز
▪ روش سطح پاسخ

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¹ substrate

² R-squared or R²

³ R²-adjusted