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Optimizing Growth Conditions of *Kluyveromyces marxianus* for Mannan Production as a Bioemulsifier

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Abstract

Background and objective: Mannan which is a linear glycoprotein with β-1,4 links carrying mannose units bind to proteins, includes natural amphiphiles and serves as a bioemulsifier. The aim of this study was optimization of growth and purification of *Kluyveromyces marxianus* for mannan production, which can use as a natural bioemulisifier.

Material and methods: In this study, mannan production by *Kluyveromyces marxianus* was assessed using combinational method of fractional factorial design and response surface methodology optimization. Process variables include concentration of carbon source (15, 30, 45 g l^{-1}) of glucose, and glycerol and methanol at 0, 2.5 and 5 gl^{-1}), nitrogen source (yeast extract and peptone 4, 6 and 8 gl^{-1}), as well as fermentation time (48, 96 and 144 h), pH (4, 6, 8) and agitation speed (150, 200 and 250 rpm).

Results and conclusion: Results showed that four variables of carbon and nitrogen source concentrations, as well as fermentation time and pH included the greatest effects on mannan production. Optimization of the affecting factors using response surface methodology demonstrated appropriate conditions of mannan production by *Kluyveromyces marxianus* as 55.15 g l^{-1} of glucose, 9.35 g l^{-1} of yeast extract, pH of 4.99 and fermentation time of 168 h, which led to a mannan yield of 245.98 mg $(100\ ml)^{-1}$culture media.

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1. Introduction

Prebiotics are non-digestible ingredients that help their hosts improve their health by increasing growth and activity of the colon bacteria [1]. Health effects associated with prebiotic consumption decrease acute gastroenteritis and cancer risk and increase mineral absorption and lipid regulation [2]. Mannan, with β-1,4 glucan, is a useful prebiotic on yeast cell surface, acting as a high-affinity ligand. Moreover, the compound is able to make binding sites [3,4].

Bioemulsifiers are active surface compounds with biological origins that can be extracellular or attached to the cell surface. This compounds are produced by bacteria yeasts and fungi. Regarding synthetic compounds, bioemulsifiers are important due to their natural origins, high biodegradability and benefits for human health. However, some bioemulsifiers described in bacteria include harmful properties that pose limitation for being widely used [1-3]. Yeasts are other sources of bioemulsifiers; however, a majority of them can produce bioemulsifiers only in the presence of water insoluble compounds such as alkanes and oils. In addition, relatively low-yields and difficulties associated with bioemulsifier extraction from growth media are other restrictions [4-6]. The mannoprotein bioemulsifier is a glycoprotein with a molecular weight of 14000-15800

Da. Inside of the cell wall of *Saccharomyces* (*S.*) *cerevisiae*, mannoprotein molecules are entrapped in glucan networks and released from cell wall of the yeast by thermal treatment under pressure. This bioemulsifier is able to stabilize oil-in-water (o/w) emulsions. Studies suggest that the chemical can be used in mayonnaise sauces with critical micelle concentration (CMC); thus, decreasing use of xanthan gums.

The baker's yeast is used for the production of this bioemulsifier due to its low costs, availability and non-toxicity. The mannoprotein molecule is composed of a polypeptide chain as well as short and long mannose links. It is stable within a broad pH range of 3-11. Extraction of mannoprotein molecules from baker's yeast cell wall is feasible using two methods of thermal and enzymatic (β1 & 3 glucanases) processes. If the protein content of mannoprotein molecules is separated by protease enzymes, the emulsifier properties of mannoprotein is lost. This bio-emulsifier is active at 5% concentrations of NaCl or higher and its production is practical at industrial scales [7-9].

In the mannoprotein structure, protein shifts into a natural amphiphilic compound through binding hydrophobic groups to mannose units; thereby, enabling its widespread use in development of food, pharmaceutical and cosmetic emulsion systems [10]. Mannoprotein was initially derived from the cell wall of *S. cerevisiae*, by Cameron et al. and Moreira et al., described as an effective polymeric bioemulsifier [11,12]. They reported higher chemical yields with no difficulties in extraction of the emulsifier from culture media as mannoprotein advantages rather than other microbial bioemulsifiers. Dikit et al. extracted and purified mannoprotein from *S. cerevisiae* KA01 by autoclaving in buffer and dialyzing in distilled water, respectively [13]. Liu et al. assessed various methods for the extraction of mannan and glucans from the cell wall of *S. cerevisiae*. They found that hot-water extraction in buffer and purification in hexadecyltrimethylammonium bromide [$C_{16}H_{33}N(CH_3)_3Br$] were the optimum methods with the greatest protective effects on mannoprotein structure [14].

In the current study, a combinational method of fractional factorial design and response surface methodology optimization was used for determining affecting factors on mannan yields in culture media. Results indicated high proportionality and confidence coefficient to predict the yields of mannan under various conditions. The optimum condition for the mannan production, optimum pH and maximum time of the appropriate yeast growth and mannan production were investigated using *Kluyveromyces* (*K.*) *marxianus* at various concentrations of carbon and nitrogen sources. Model and synthetic data from this study provide basic and functional information to estimate the substrate use, product formation and operation design, especially in industrial settings.

2. Materials and methods

All chemicals were purchased from Merck, Germany.

2.1. Yeast strains and inoculum stock preparations

K. marxianus IBRC-M 30114 was provided by Iranian Biological Resource Center. After recovery, cells were stored at 4°C on yeast-peptone-dextrose agar slants. To prepare 100 ml of the culture media, peptone (2 g), glucose (2 g) and yeast extract (1 g) were mixed in a tube and then sufficient amount of double distilled water was added to the mixture.

2.2. Inoculum development

A loopful of the yeast colony was transferred into an Erlenmeyer flask containing 100 ml of the inoculum media under sterile conditions. Flask was then incubated with agitation using shaking incubator (Orbital Shaker Incubator Rs 25000, India) at 180 rpm for 24 h at 28°C [15-17].

2.3. Culture media preparation

Briefly, 1600 ml of the yeast-peptone-dextrose culture media were modified in an Erlenmeyer flask using peptone (32 g), glucose (32 g), yeast extract (16 g) and 1600 ml of double distilled water The final solution was divided into 19 Erlenmeyer flasks. The pH of each flask was adjusted to certain values using concentrated HCl or 4.0 N NaOH solution. Then, flasks were autoclaved at 121°C for 20 min at 1.2 bar.

2.4. Manufacturing condition

Variables, including type and concentration of the carbon and nitrogen sources, pH, time, stirring speed and yeast type, were assessed according to a full factorial design (Table 1).

To prepare culture media, compounds such as KH_2PO_4 (3 g l^{-1}), $(NH_4)_2SO_4$ (4 g l^{-1}), $MgSO_4.7H_2O$ (0.5 g l^{-1}), $CaCl_2$ (0.13 g l^{-1}), and NaCl (0.2 g l^{-1}) were used. Totally, 300 µl of the inoculum were transferred to each flask. Culture media were incubated at 30°C for 48 and 144 h at 150-250 rpm using shaker incubator [18].

2.5. Isolation and preparation of the yeast cells

Cells were isolated using centrifugation of the culture media at various speeds for a certain duration of time. After rinsing with cool deionized water, cells were agitated vigorously using vortex mixer with glass beads in ice. After removing and washing the glass beads, suspensions were centrifuged at appropriate speeds for a certain duration of time. Precipitated cells were rinsed several times using cool deionized water [19,20].

2.6. Extraction and purification of mannan

Yeast cell precipitates from the previous step were resuspended in 20% w w^{-1} buffer solution, (0.1 M potassium citrate and 0.02 M sodium metabisulfite, pH 7.0) and autoclaved at 121°C for 90 min at 1.2 bar. The mannoprot-

ein was washed with acetic acid (1%) in ethanol (96%) and precipitated using centrifugation at 748 ×g for 10 min. The supernatant was stored at 4°C overnight and then centrifuged at 7656 ×g for 10 min to complete the precipitation [19,21,22]. Hexadecylt rimet hylammonium bromide chemical [$C_{16}H_{33}N$ $(CH_3)_3Br$] was used as solvent for selective precipitation and purification of the mannan from other macromolecules such as proteins. To complete the purification process, the precipitates were dialyzed against deionized water for 48 h [23-25].

2.7. Biomass quantity assessment

Yeast cells were isolated from suspensions using centrifugation at 748 ×g for 10 min. Then, cells were collected and dried at 105°C for 5 h until reaching a constant weight [25,26].

2.8. Extraction and purification yields

The compound yields were calculated as percent of the dry weight of the extracted mannan in 100 ml of the yeast culture media [27-29].

2.9. Experimental design and data analysis

Concentration level and effect range of each variable were assessed as pretreatments using single factor at a time. To select the most important variables, fractional factorial design was used with eight treatments (16 treatments and three center points) (Table 1). Optimization of the significant variables was carried out using response surface methodology based on the central composite design and Minitab Software v.17 or design of experiments. Microsoft Excel v.16 was used to draw charts.

3. Results and discussion

3.1. Selection of the carbon source

First, various carbon sources (glycerol and methanol) were assessed based on the highest yields of mannan and cell biomass in a limiting solution of saturated glucose (Table 2). Data analysis showed a significant difference in carbon sources. The maximum quantity of mannan produced in the presence of glucose by *K. marxianus* included 205±4.4 mg in 100 ml of culture media with a significant difference, compared to other sources. The mannan yields in the presence of carbon sources (methanol and glycerol) were significantly lower than that in presence of others ($P<0.05$). Use of glucose as carbon source was reported as the best choice of carbon source ($P<0.05$) for mannan production.

3.2. Selection of the nitrogen source

A variety of nitrogen sources (organic) were first assessed based on the highest yields of mannan and cell biomass in solutions of carbon sources (Table 3). A significant difference was observed between various sources. The maximum quantity of mannan produced in the presence of yeast extract by *K. marxianus* included 142 ± 3.3 mg per 100 ml. Effects of various nitrogen sources on mannan yields were as follows: the yield of the production when utilizing extract was statistically more than extract and peptone together and peptone alone.

Table 1. Variable levels presented by real data or codes in fractional factorial design

Level variables	Carbon Sources			Nitrogen Sources		Environmental condition		
	Glucose	Glycerol	Methanol	Yeast Extract	Peptone	pH	Time	Agitation Speed
Coded	g l^{-1}	g l^{-1}	g l^{-1}	g l^{-1}	g l^{-1}		h	rpm
1	15	0	0	4	4	4	48	150
0	30	2.5	2.5	6	6	6	96	200
-1	45	5	5	8	8	8	144	250

Table 2. Effects of adding different carbon sources on the yields of mannan and biomass of *K. marxianus*

Nitrogen resources	Mannan mg (100 ml)$^{-1}$	Biomass (g l^{-1})
Glycerol	131±2.3^{a*}	12.24±0.15
Methanol	45±3.5^{b}	3.04±0.20
Glycerol and methanol	91±1.6^{c}	8.36±0.11
Glucose	205±4.4^{d}	17.95±0.30

*Results are statistically significant ($P\leq0.05$) in each column with non-similar alphabets

Table 3. Effects of adding various sources of nitrogen to culture media on the yields of mannan and biomass of *K. marxianus*

Nitrogen resources	Mannan mg (100 ml)$^{-1}$	Biomass (g l^{-1})
Yeast Extract	142±3.3^{a*}	12.88±0.21
Peptone	112±4.6^{bc}	10.74±0.37
Yeast Extract+Peptone	129±2.5^{b}	11.55±0.30

*Results are statistically significant ($P\leq0.05$) in each column with non-similar alphabets

Similar findings were reported by Liu et al. for *S. cerevisiae*. They achieved the highest yields when organic nitrogen sources such as soy peptone and yeast extract were available. This revealed that inorganic nitrogen sources included no significant effects on increasing the mannan yields [24]. similar to the results by Demirci and Pometto, organic nitrogen sources enable yeasts to increase cell biomass and cell wall thickness due to easy transfer and adsorption of these sources [30]. Similar to the present results for *K. marxianus*, Mohammadzadeh et al. demonstrated that presence of organic nitrogen sources such as peptone and yeast extract led to the highest yields [31].

3.3. Screening of the process parameters

The major aim of this assessment was to select the most important variables affecting mannan production using fractional factorial design under specific conditions. Analysis of variance demonstrated that the first-order model was appropriate to fit the main effects of the six variables. Estimates of the coefficients and associated P-values within 95% confidence interval (CI) are summarized in Table 4. Mannan production and cell biomass were significantly affected by the concentration of carbon source (glucose), concentration of yeast extract, time and pH, respectively. Glycerol (as an enzyme activating agent), peptone and mixing speed included positive effects on mannan production. Although their coefficients were as important as other factors, the fixed upper limits were considered for the optimization. Bzducha-Wrobel et al. reported positive effects of these factors as accelerators of the mannan biosynthesis in the cell wall structure of *S. cerevisiae*. Effects of methanol addition on mannan production and cell biomass seemed not significant. Thus, concentration of the carbon sources, fermentation time and pH were used as the major variables for the optimization using response surface methodology [32].

3.4. Optimization of the mannan production

To show possible changes in mannan yields and assess effects of the independent variables, including carbon and nitrogen sources, pH and fermentation time, the most appropriate model should be used to fit data. As for the prediction of yields, linear and second-order polynomial models were fitted to data (Table 5). Based on R^2 (0.996), modified R^2 (0.994) and non-significant results from lack of fit test, the second-order polynomial model was chosen. The fitted second order model was statistically significant ($P<0.05$) and lack of fit test was non-significant for all attributes at 95% level (after model fitting, the resultant relation was subjected to the backward algorithm to reduce non-significant terms in the model).

Table 4. Estimated effects of the major variables on mannan production resulted from fractional factorial design

Source	DF	Adj SS	Adj MS	F-value	P-value	Effect	Coefficient	SE Coefficient
Model	16	61604.6	3850.3	1898.16	0.001			
Linear	8	35854.3	4481.8	2209.48	0.000		102.361	0.356
Glucose	1	3333.2	3333.2	1643.26	0.001	-28.867	-14.434	0.356
Glycerol	1	155.3	155.3	76.57	0.013	6.231	3.116	0.356
Methanol	1	0.0	0.0	0.01	0.921	0.080	0.040	0.356
Yeast Extract	1	669.9	669.9	330.25	0.003	12.941	6.471	0.356
Peptone	1	300.6	300.6	148.22	0.007	8.670	4.335	0.356
pH	1	3259.8	3259.8	1607.03	0.001	-28.547	-14.274	0.356
Time	1	26110.0	26110.0	12871.98	0.000	80.793	40.396	0.356
Agitation	1	2025.5	2025.5	998.56	0.001	22.503	11.251	0.356
2-Way Interactions	7	3019.7	431.4	212.67	0.005			
Glucose*Glycerol	1	400.8	400.8	197.61	0.005	-10.010	-5.005	0.356
Glucose*Methanol	1	19.3	19.3	9.50	0.091	-2.195	-1.098	0.356
Glucose*Yeast Extract	1	107.6	107.6	53.02	0.018	-5.185	-2.593	0.356
Glucose*Peptone	1	12.2	12.2	6.04	0.133	1.750	0.875	0.356
Glucose*pH	1	255.4	255.4	125.93	0.008	7.991	3.996	0.356
Glucose*Time	1	1277.9	1277.9	629.98	0.002	-17.874	-8.937	0.356
Glucose*Agitation	1	946.4	946.4	466.59	0.002	-15.382	-7.691	0.356
Curvature	1	22730.6	22730.6	11205.97	0.000			
Error	2	4.1	2.0					
Total	18	61608.6						
		S	R-sq	R-sq (adj)				
		1.42423	99.99%	99.94%	*			

Table 5. Central composite design for the independent variables at each run (real data or codes)

Run order	Glucose (g l^{-1})	yeast extract (g l^{-1})	pH	Time (h)	Mannan mg (100 ml)$^{-1}$	Biomass (g l^{-1})
1	45	4	4	48	68.35	6.34
2	30	6	6	96	180.44	16.99
3	45	4	8	144	187.27	17.86
4	15	8	8	144	109.20	10.04
5	15	8	4	48	74.06	7.27
6	15	4	8	48	58.88	5.66
7	15	4	4	144	90.37	8.39
8	30	6	6	96	179.25	16.85
9	45	8	4	144	224.80	18.90
10	45	8	8	48	111.27	10.60
11	15	8	8	48	64.55	6.00
12	45	4	8	48	81.65	7.73
13	15	8	4	144	107.22	10.12
14	30	6	6	96	180.70	17.47
15	15	4	4	48	35.51	3.80
16	45	8	8	144	184.70	17.15
17	45	8	4	48	129.10	11.36
18	15	4	8	144	108.48	9.50
19	30	6	6	96	179.21	16.77
20	45	4	4	144	194.20	17.38
21	30	2	6	96	137.66	11.92
22	30	10	6	96	189.71	17.24
23	30	6	10	96	62.68	5.85
24	30	6	2	96	81.42	7.04
25	30	6	6	0	38.50	3.59
26	60	6	6	96	171.75	17.02
27	30	6	6	192	184.24	17.1
28	0	6	6	96	35.30	3.05
29	30	6	6	96	180.83	16.64
30	30	6	6	96	180.15	16.90

Table 6 describes effects of the major factors and second order effect of the factors on the mannan yields. It was found that the mutual interactions of these tested factors such as glucose concentration and yeast extract concentration, time and pH, yeast extract concentration and pH, and time and pH were significant, with carbon source concentration and time, nitrogen source concentration and pH, and nitrogen source concentration and time showing the greatest effects on response. Furthermore, R^2 and modified R^2 were relatively similar, indicating that non-significant variables were not added to the model. High R^2 and modified R^2 values as well as proportionality of these two parameters verified strength of the model to predict outcomes [33]. To determine the surface response model, linear and second-order responses and mutual interactions between the independent variables were used. The following equation demonstrated experimental relationships between the mannan yields and the tested variables within the real data:

$$\text{Mannan (mg (100 ml)}^{-1}) = -473.5 + 5.810\ X_1 + 30.03\ X_2 + 2.088\ X_3 + 97.27\ X_4 - 0.08383\ X_1^2 - 0.955\ X_2^2 - 0.007335\ X_3^2 - 6.682\ X_4^2 + 0.1179\ X_1X_2 + 0.01895\ X_1X_3 - 0.1781\ X_1X_4 - 0.0579\ X_2X_3 - 1.770\ X_2\ X_4 - 0.0236\ X_3\ X_4 \quad \text{Equation (1)}$$

3.5. Effects of the carbon source concentration and fermentation time on mannan yields

Effects of the carbon source concentration and time on mannan production yield under condition; pH=6 and yeast extract concentration=6 g l^{-1}, was presented as contour plot (Figure 1).

3.6. Effects of the nitrogen source concentration and pH on mannan yields

Effects of the yeast extract concentration and pH on mannan production yield after 96 h and glucose concentration equal to 30 g l^{-1} was presented as contour plot (Figure 2).

Increasing yeast extract concentration up to 9.65 g l^{-1} within a pH range of 5.2 to 5.43 resulted in increasing of the mannan yields. The pH values greater than 5.43 included no positive effects on the mannan yields. Concentrations of more than 9.65 g l^{-1} were constant. It seemed that yeasts failed to adsorb higher concentrations of the yeast extract because the carbon source was used and growth was suppressed. Low concentrations of the nitrogen source decreased protein biosynthesis and hence cell wall proteins. In contrast, high concentrations increased the growth rate but inhibited mannan production due to the complex nature of such sources. Lizicarova et al. concluded that deficient nitrogen sources decreased effects on cell growth and mannan by 14%, similar to the current findings. Therefore,

yeast extract concentrations between 10 and 11 g l^{-1} and pH values of 5-5.5 produced the highest mannan yields [34].

Table 6. Analysis of the effect of variables and mutual interactions between independent variable on the responses

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	16	97239.0	6077.4	221.40	0.000
Blocks	2	27.4	13.7	0.50	0.618
Linear	4	62413.8	15603.5	568.43	0.000
Glucose(g l^{-1}) (X_1)	1	27066.2	27066.2	986.02	0.000
Yeast Extract (g l^{-1}) (X_2)	1	3367.5	3367.5	122.68	0.000
Time (h) (X_3)	1	31853.7	31853.7	1160.43	0.000
pH (X_4)	1	126.5	126.5	4.61	0.050
Square	4	29781.6	7445.4	271.24	0.000
Glucose^2 (g l^{-1}) (X_1^2)	1	9757.1	9757.1	355.45	0.000
Yeast extract^2 (g l^{-1}) (X_2^2)	1	400.4	400.4	14.59	0.002
Time^2 (h) (X_3^2)	1	7833.4	7833.4	285.37	0.000
pH^2 (X_4^2)	1	19596.8	19596.8	713.91	0.000
2-Way Interaction	6	5016.2	836.0	30.46	0.000
Glucose(g l^{-1})*yeast extract: X_1 X_2	1	200.3	200.3	7.30	0.018
Glucose(g l^{-1})*Time: X_1 X_3	1	2979.2	2979.2	108.53	0.000
Glucose(g l^{-1})*pH : X_1 X_4	1	457.0	457.0	16.65	0.001
Yeast extract (g l^{-1}) *Time(h): X_2 X_3	1	495.0	495.0	18.03	0.001
Yeast extract (g l^{-1})*pH : X_2 X_4	1	802.4	802.4	29.23	0.000
Time(h)*pH : X_3 X_4	1	82.2	82.2	3.00	0.107
Error	13	356.8	27.4		
Lack-of-Fit	10	354.8	35.5	51.94	0.004
Pure Error	3	2.0	0.7		
Total	29	97595.9			
		S	R-sq	R-sq (adj)	R-sq (pred)
		5.23926	99.63%	99.18%	97.08%

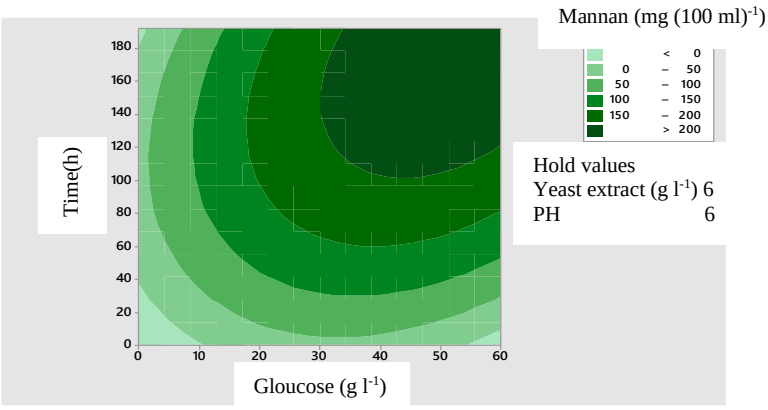

Figure 1. Contour plot of the effects of carbon source and time on the yields of mannan extracted from *Kluyveromyces marxianus*

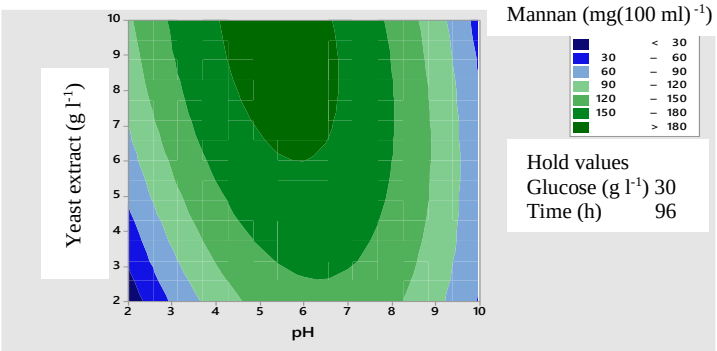

Figure 2. Contour plot of the effects of nitrogen source and pH on the yields of mannan extracted from *Kluyveromyces marxianus*

3.7. Effects of the nitrogen source concentration and fermentation time on mannan yields

Effects of the yeast extract concentration and time on mannan yields under conditions; pH=6 and glucose concen

tration 30 g l^{-1} was presented as contour plot (Figure 3).

Increased yeast extract concentrations up to 9.5 g l^{-1} with time periods of 121 and 161 h were associated to the increased yields. Times greater than 161 h included no positive effects on yields. Concentration more than 9.5 g l^{-1} did not change. It seemed that yeasts failed to adsorb higher concentrations of the yeast extract because the carbon source was used and growth was suppressed. Low concentrations of the nitrogen source decreased the protein biosynthesis and hence the cell wall proteins. High concentrations increased the growth rate but inhibited mannan production due to the complex nature of such sources.

3.8. Effects of the carbon source concentration and pH on mannan yields

Relationships between the factors and the responses can be well understood using contour plot when considering functions of the two variables simultaneously and fixing other variables at midpoints. Effects of the carbon source concentration (e.g. glucose) and pH on mannan yields under the conditions of time of 96 h and yeast extract concentration of 6 g l^{-1} was represented as contour plot (Figure 4). Since the linear and second-order interactions between the glucose and the pH were significant, curvatures occurred in the plots. Increases in glucose concentrations up to 42.88 g l^{-1} led to significant increases in yields, particularly at pH values of 5.5-5.7. From this concentration to concentration of 43 g l^{-1}, yields were constant; higher concentrations included decreasing effects on the yields.

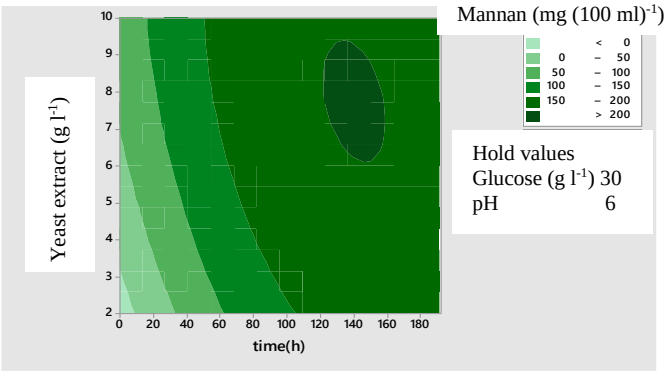

Figure 3. Contour plot of the effects of nitrogen source and time on the yields of mannan extracted from by *Kluyveromyces marxianus*

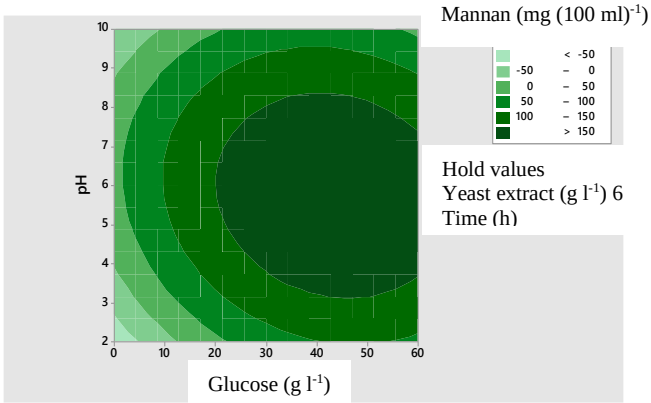

Figure 4. Contour plot of the effects of carbon source and pH on the yields of mannan extracted from by *Kluyveromyces marxianus*

It was indicated that the mannan yields sharply decreased at low pH values. However, even increases in other factors such as glucose concentration could not increase the mannan yields, needing low rates of glucose consumption as substrate of the fermentation media. Therefore, it can be concluded that the mannan yields were high at glucose ranges of 42-43 g l^{-1} and pH values of 5.5-5.8. Out of these ranges, increases in pH and glucose concentrations included no positive contributions to the mannan yields. Francois and Aguilar-Uscanga reported that roles of the cell wall and its composition varied fundamentally depending on type and concentration of the carbon sources [35]. Liu et al. reported 49 g l^{-1} of sucrose as the maximum yield of mannan production by *S. cerevisiae*. They showed that high carbon source concentrations decreased the cell biomasses and mannan yields because of variable ionic strengths and great osmotic pressures [24]. In a study by Lukondeh et al., it was shown that glucose concentrations of 60 g l^{-1} and greater decreased cell biomasses in *K. marxianus* [36]. Another inhibitory parameter to high sugar concentrations includes production

of ethanol during biomass expansion, which decreases cellular growth [37,38].

3.9. Effects of pH and fermentation time on the mannan yield

Effects of pH and time on mannan yields under the conditions of yeast extract concentration of 6 g l^{-1} and glucose concentration of 30 g l^{-1} was represented as contour plot (Figure 5).

Time periods up to 161 h contributed to improvements of the mannan yields. Decreases in cellular growth and mannan was seen at time periods greater than 161 h. The lower the time periods, the lower the mannan yields. Increases in other factors such as the carbon source concentration could not improve the mannan yields, suggesting low rates of glucose consumption at this condition. Time periods of 149-161 h and pH values of 5.4-6.1 demonstrated the highest mannan yields.

Time is one of the most important environmental factors, affecting growth of the microorganisms and accounting for many changes in biosynthetic and metabolic pathways. Hence, it is expected that time increases or prevents the growth of certain metabolites. Liu et al. demonstrated the maximum yields of mannan in *K. marxianus* at 96 h and 28°C. They reported that the yeast growth and activity of the mannan biosynthetic enzymes were inhibited at time periods greater than 120 h [24]. Aguilar and Francois assessed effects of pH and temperature on the composition of cell walls, especially mannan. Increases in pH from 3 to 5 significantly increased mannan production, followed by a decrease [35]. Schultz et al. reported the maximum biomass of *K. marxianus* from whey concentrates at pH 5.6 and 33°C. Their results verified the current range of pH and constant temperature (30°C) [39].

3.10. Optimization and validation of the model

The optimal condition for the mannan production by *K. marxianus* included 55.15 g l^{-1} of glucose concentration, 9.35 g l^{-1} of yeast extract concentration, pH 4.99, temperature of 30°C and time of 168 h, which resulted in the maxim yield of 245.98 mg (100 ml)$^{-1}$. To validate the model, Erlenmeyer flask experiments at optimal points were carried out in triplicate and the experimental yields were compared with the predicted yields from the model. The mean yield was 2220 ±18.0 mg l^{-1}. Considering the predicted yield from the model at optimal point for two yeasts [e.g. 2230 mg l^{-1}], it seemed that the predicted and experimental yields were relatively similar. Moreover, carrying out the experiments in batch fermentation under the optimum yields could reach 2459.8 mg l^{-1}. Compared to Erlenmeyer flask experiments, this improvement in mannan yields in fermentation could be attributed to the increased quantity of available oxygen, uniform distribution of nutrients and better control of pH in culture media [40,41].

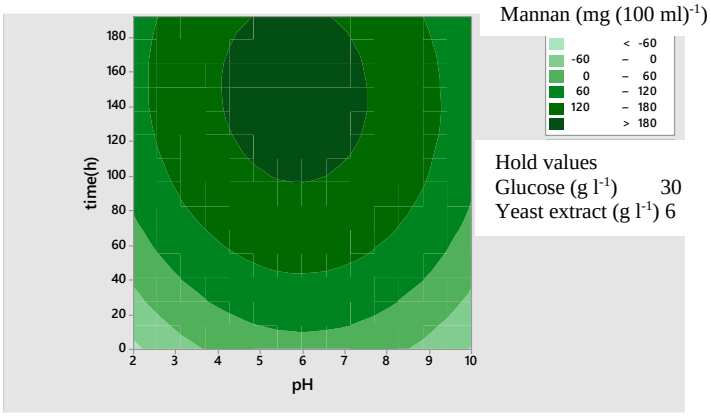

Figure 5. Contour plot of the effects of pH and time on yields of mannan extracted from by *Kluyveromyces marxianus*

3.11. Kinetics of cell growth and mannan production at optimal conditions

Kinetic studies are important for optimizing biologic processes. Studying curves of growth and mannan production yeild revealed that glucose was completely consumed and yeast entered the stationary phase after 30 h. The highest quantity of mannan was achieved at the beginning of this phase (Figures 6a, b). When the carbon source was used, the yeast growth was prevented at the end of the process, showing that glucose was appropriate for the yeast and was the only limiting substrate for the microbial growth in the media. During the exponential phase, the ratio of special growth to time was constant. This suggest that no inhibitory effects such as alcohol were present in the media [42-44].

Mannan can be extracted from several yeast strains, representing 4-13% of the cell dry weight content. Similar findings by Ozmihci and Kargi were reported on kinetic models of the *K. marxianus* growth at various concentration of the carbon source [45].

Galinari et al. and Lukondeh et al. reported kinetic characteristics of *K. marxianus* with yield of the purified bioemulsifier (7-13.3%) of the dry cell weight and concentration of the purified product (12 g l^{-1}) [29,36].

Solis-Pacheco et al. reported the kinetic characteristics of *S. cerevisiae* (CTGM, CTSA) and *Meyerozyma guilliermondii* (CT15, CT25, CT35) with 30 g l^{-1} of sugars and 5 g l^{-1} of $(NH_4)_2SO_4$, they found that the cell wall composition was more variable between the strains than the species and was highly depended on the growth phases. In the stationary phase, only two strains (CT15 and CT25) included high β-glucan and mannan contents in the cell wall (~30 mg g^{-1}) while the others yielded 3-18 mg g^{-1} as well as control bakery yeast strain [46].

4. Conclusion

The increasing popularity of emulsifier consumptions has resulted in development of novel sources to produce bioemulsifiers. Biological approaches serve as the most effective and appropriate tools for producing these products using microorganisms.

Results of this study have represented a successful model for optimizing conditions that affect the production yields of mannan extracted from *K. marxianus*. Furthermore, data from this study have provided basic information for further investigations of mannan as a safe and effective bioemulsifier for using in food formulations and pharmaceutical products at large scales.

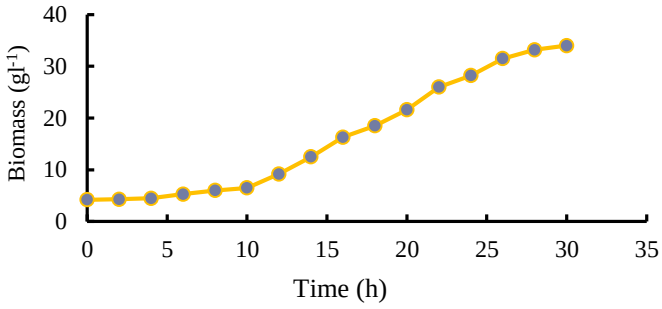

Figure 6a. The growth curve of *Kluyveromyces marxianus* at optimal conditions

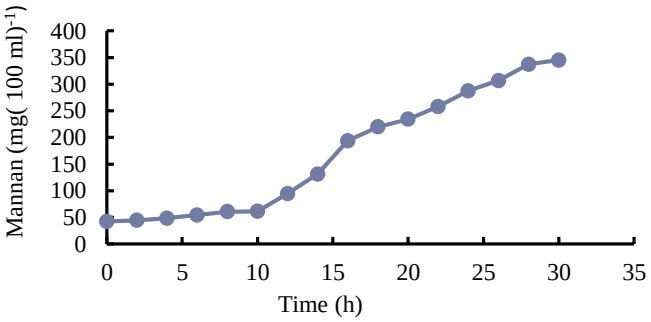

Figure 6b. The yield curve of mannan extracted from *Kluyveromyces marxianus* under optimal conditions

Table 7. Conditions of the batch fermentation system for the process of *K. marxianus* growth during 30 h

Time	rpm	O_2	CO_2	pH	Do	Temp. (°C)	Biomass (g l^{-1})	Mannan mg (100 ml)$^{-1}$
12:30	500	20.3	0.05	4.31	32.5	27.3	4.1	42.5
18:00	500	19.7	0.15	4.66	29.1	28.9	6.5	61.3
20:30	500	19.5	0.21	4.59	25.8	29.3	9.2	94.6
22:30	500	19.3	0.25	4.51	20.9	29.4	12.5	131.2
0:30	500	19.5	0.23	4.56	26.5	29.1	16.3	193.6
2:30	500	19.4	0.24	4.57	25.8	29.2	18.5	220.0
4:00	500	19.5	0.26	4.57	26.7	29.1	21.6	234.3
6:30	500	19.5	0.24	4.58	31.2	29.1	26	257.9
8:00	500	19.6	0.23	4.61	32.7	28.9	28.2	287.5
10:30	500	19.6	0.21	4.60	33.2	28.9	31.5	306.5
12:00	500	19.1	0.13	4.60	35.2	29.2	33.2	337.2
14:30	500	19.7	0.06	4.60	36.5	29.1	34	345.1

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6. Conflict of interest

The authors declare no conflict of interest.

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بهینه سازی شرایط رشد *کلایورومایسس مارکسیانوس* برای تولید مانان به‌عنوان بیوامولسیفایر

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چکیده

سابقه و هدف: مانان، گلیکو پروتئینی خطی با اتصالات بتا ۱-۶ متشکل از واحد های مانوز متصل به پروتئین است که دارای ساختار ترکیبات آمفی فیلیک طبیعی بوده و می تواند به عنوان بیوامولسیفایر مورد استفاده قرار گیرد. هدف از این مطالعه، بهینه سازی شرایط رشد و خالص سازی *کلایورومایسس مارکسیانوس* برای تولید مانان است که به عنوان یک بیوامولسیفایر طبیعی قابل استفاده است.

مواد و روش‌ها: در این مطالعه، تولید مانان توسط *کلایورومایسس مارکسیانوس* با استفاده از ترکیب طرح کسری از فاکتوریل و سطح پاسخ مورد بررسی قرار گرفت. متغیرهای تحقیق شامل غلظت منبع کربن (گلوکز در غلظت‌های ۱۵، ۳۰ و ۴۵ gl^{-1} و نیز گلیسرول و متانول در غلظت‌های ۰، ۲/۵ و ۵ gl^{-1})، منبع ازت (عصاره مخمر و پپتون در غلظت‌های ۴، ۶ و ۸ gl^{-1})، زمان تخمیر (۴۸، ۹۶ و ۱۴۴ h) و pH (۴، ۶ و ۸)، سرعت همزن (۱۵۰، ۲۰۰ و ۲۵۰ rpm) بود.

یافته‌ها و نتیجه‌گیری: نتایج نشان داد چهار متغیر غلظت منبع کربن و نیتروژن، زمان تخمیر و pH بیشترین تاثیر را بر تولید مانان داشتند. بهینه سازی عوامل موثر با روش سطح پاسخ، شرایط مناسب تولید مانان از *کلویورومایسس مارکسیانوس* را با غلظت‌های ۵۵/۱۵ گرم بر لیتر گلوکز، ۹/۳۵ گرم بر لیتر عصاره مخمر، pH برابر ۴/۹۹ و زمان تخمیر ۱۶۸ساعت نشان داد که به راندمان تولید مانان به میزان ۲۴۵/۹۸ $mg(100ml)^{-1}$ محیط کشت منجر شد. بنابراین نتایج این تحقیق می تواند به عنوان یک الگوی موفق در بهینه سازی عوامل مؤثر در راندمان تولید مانان در محیط کشتی از *کلایورومایسس مارکسیانوس* استفاده شود.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- مانان
- تولید

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