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Response surface optimization of the cultivation conditions and medium composition a novel probiotic strain *Bacillus pumilus* STF26

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Abstract

Probiotics have been widely used in aquaculture as a controlling disease, enhancing immune responses, providing nutrients, and improving water quality. The use of *Bacillus* species as probiotics is expanding rapidly with increasing number of studies. Optimization of cultivation conditions and the medium composition for the improvement of novel potential probiotic strain *Bacillus pumilus* STF26 was performed by using response surface methodology (RSM). Factors optimized were temperature, pH and the concentrations of dextrose, yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$. The optimum values are found as 30.9°C, 6.9, 20% (w/v), for temperature, pH and the concentrations of dextrose and 1.526% (w/v), 0.1% (w/v) and 0.5% (w/v), for yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$, respectively. As a result, maximum biomass at optimum conditions was 10.42 g/L, which is nearly 2.5 times higher when compared to the one obtained by using Luria Broth medium at optimized temperature and pH values.

Introduction

The widespread and intense use of antibiotics for therapeutic purposes has led to a considerable increase in the number of antibiotic-resistant bacteria, resulting in occurrence of serious and hard-to-treat infections in both humans and livestock (Barbosa and Levy, 2000; Barbosa *et al.*, 2005; Chaiyawan *et al.*, 2010). Therefore, there has been an increasing concern about the use of antibiotics and they are not permitted to be used as feed additives in livestock (Patterson and Burkholder, 2003; Foerst *et al*., 2012). Thus, researchers and feed companies have started a search for alternative products to prevent and control infectious diseases (Chaiyawan *et al.*, 2010; Santini *et al.*, 2010). An effective and safe alternative to antibiotic implementation is the use of probiotics which protect the animal from pathogens by improving the microbial balance in the gastrointestinal tract to exclude potentially harmful bacteria (Modesto *et al.*, 2009; Gaudana *et al.*, 2010; Gupta *et al.*, 2011).

Probiotics are live microorganisms, which are favorable to their host, when administered in adequate amounts (Lutful Kabir, 2009; Cutting, 2011). Probiotics influence the health of host by preventing the growth of pathogenic microorganisms, improving the intestinal microbial balance thereby leading to improved nutritional absorption, promoting digestion and feed intake and inducing the immune system (Duc *et al.*, 2004; Kim *et al.*, 2009). Therefore, the use of probiotics on livestock enhances the growth of animals, improves efficiency of feed conversion and decreases the rate of mortality (Kosin and Rakshit, 2006). Probiotic microorganisms should be non-pathogenic, non-toxic and should improve growth of the host animal. In addition, probiotic microorganisms should be able to survive and continue their metabolic activities in gastrointestinal conditions and produce compounds that inhibit the growth of pathogenic microorganisms (Patterson and Burkholder, 2003; Kim *et al.*, 2009).

The most common probiotic species used in humans are *Lactobacillus* and *Bifidobacterium* species, while *Bacillus*, *Enterococcus*, and *Saccharomyces* species are mostly used in livestock. Among those, *Bacillus* species are more preferable because they are endospore-formers, have extreme resistances to heat, chemicals, and other stresses (Nicholson *et al.*, 2000; Setlow, 2006; Cartman *et al*., 2008). *Bacillus* spores can survive in harsh pH conditions of the gastric fluids (Cutting, 2011) and reach the small intestine, making them better suitable for use as feed supplements. However, there is always a need for effective and novel probiotic strains with

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high antimicrobial activity. Some studies have demonstrated that Bacillus pumilus act as probiotic by promoting the growth and viability of the beneficial lactic acid bacteria in the intestinal tracts of humans and some animals (Ghosh *et al.*, 2002; Aly *et al*., 2008). For these reason, optimum growth conditions and medium composition should determinate and can be utilized for commercial production.

RSM is a statistical technique, which is used to build an empirical model relating a response and the factors that affect (Montgomery, 1984; Box and Norman, 1987; Deming and Stephen, 1987). However, the ultimate goal of the RSM is to optimize the operating conditions of a system or to determine the region where operating conditions are satisfied. RSM is widely and successfully used in optimization of media composition and process parameters for microorganism growth (Preetha *et al.*, 2007; Fung *et al.*, 2008; Saelao *et al*., 2011).

Considering the lack of any reports investigating optimization of growth media composition and cultivation conditions of a novel probiotic strain, *Bacillus pumilus* STF26, this is the first report investigating this subject. Factors optimized were temperature, pH and the concentrations of dextrose as carbon source, yeast extract as a nitrogen source, KH_2PO_4 and $MgSO_4.7H_2O$ using response surface methodology. The major objective of this study was to investigate the optimum growth conditions and medium composition for commercial production.

Materials and Methods

A potential probiotic microorganism, *Bacillus pumilus* STF26 strain was used in this study, which was isolated from bovine chyme (Ozkan, 2012) and it has high antimicrobial activity against a number of bacteria including *Salmonella enterica*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (El-Refai *et al.*, 2005).

The strain was streaked on LB agar (Sigma, United States) and stored at 4ºC to maintain viability. Stock culture was maintained at -80°C in 30% (v/v) glycerol and cultured in LB broth incubated for 24 hr at 37°C, 125 rpm. For bioreactor studies, 200 µL of 24 hr-grown culture was inoculated to 20 mL LB broth and incubated for 24 hr at 37°C, 125 rpm, which was then used as the inoculums. A Sartorius Biostat B Plus bioreactor (Germany) equipped with a 5-L vessel (2-L working volume) was used for the study.

The cultivation medium used in this study consisted of dextrose (Roquette Frères, France), yeast extract (Sigma-Aldrich, United States), KH_2PO_4 (Sigma-Aldrich, United States) and $MgSO_4.7H_2O$ (Sigma-Aldrich, United States). As the first optimization step, temperature, pH, and concentration of dextrose were varied according to the Box-Behnken design of surface response methodology. The amount of yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$ were constant for the step as 20, 2 and 1 g/L, respectively. pH was measured by using a pH electrode (Hamilton, United States) and adjusted by adding 4 N NaOH or 1 N HCl solutions by using peristaltic pumps.

In the second optimization step, temperature, pH, and concte me entration of dextrose were the optimum values obtained from the first optimization, while the concentrations of yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$ were varied according to the experimental design. For both the first and second optimization steps, agitation speed was adjusted to 200 rpm throughout the experiments. Aeration was performed by using sterile air and the flow rate was set at 2 vvm by using a rotameter (Q-flow, Vögtlin Instruments, Germany). Dissolved oxygen (DO) concentration was first adjusted to 100% saturation before inoculation and then cascaded to O_2 enrichment to prevent the drop of DO to value less than 50% saturation. DO was measured by using a dissolved oxygen sensor (Hamilton, United States). A silicone-based antifoam agent (Antifoam A concentrate, Sigma-Aldrich) was used to prevent foaming during the process. Each condition as suggested by Box-Behnken design of surface response methodology was evaluated for 30-hr batch fermentations during which samples were taken periodically.

Among the salts KH_2PO_4 and $MgSO_4.7H_2O$ are commonly used in growth medium for *B. pumilus* (Feng *et al*., 2001; Joo and Chang, 2005; Brinques *et al*., 2010; Rajendran and Thangavelu, 2010). Moreover, studies show that carbon and nitrogen concentrations, pH and temperature together with the salts have significant effects on the growth of microorganisms (Richard and Margaritis, 2003; Liew *et al.*, 2005; Das *et al.*, 2010; Turhan *et al.*, 2010). Therefore, in this study the aim is to maximize the biomass by optimizing concentrations of KH_2PO_4, $MgSO_4.7H_2O$, glucose and nitrogen sources, pH and temperature by the Box-Behnken design of surface response methodology. The advantage of this method is the reduced number of experiments with reduced replicates (Cutting, 2011).

Therefore, Box-Behnken response surface method was used in the optimization of key factors to maximize the growth of the probiotic strain. Minitab (Version 16; Inova ltd. Co.) statistical software was used to design the conditions for biomass

production by giving the minimum and maximum values of determined factors. The quality of the fit of the regression model equations was given by the coefficients of determination (R^2). The quadratic model equation was maximized by using the same software to determine the optimum levels of the variables for maximum biomass (g/L).

In first optimization, fifteen experiments were generated for three factors; temperature, pH and concentration of the carbon source. Fifteen more runs were generated for the second optimization for the concentrations of nitrogen source, KH_2PO_4 and $MgSO_4.7H_2O$. The variables for two optimizations and the coded and uncoded values were coded according to the following regression equation:

$$x_i = X_i - X_0 / \Delta X_i \quad (1)$$

where x_i is the coded value, X_i is the actual value of the independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value.

In our regression models for both of the optimizations, the response was the biomass (g/L) and the α-level at which every term in the selected model should be significant was set as 5%. Full quadratic models, used to fit the response in Box-Behnken design, were expressed as follows:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\, \beta_{ij} x_i x_j \quad (2)$$

where Y is the predicted response, β_0 is the constant, β_i is the coefficient for the linear effect, β_{ii} is the coefficient for the quadratic effect and β_{ij} is the coefficient for the interaction effect.

In order to verify the validity of the model, validation experiments were conducted at the determined optimum conditions. For the validation of the model constructed after first optimization, the parameters namely temperature, pH and concentration of carbon source (dextrose) were set at optimum levels found after statistical analyses. Likewise, in order to confirm the validity of the model generated after second optimization, concentrations of nitrogen source (yeast extract), KH_2PO_4 and $MgSO_4.7H_2O$ were set at optimum values. Biomass obtained after these experiments was compared with the one estimated by using the model equations.

The optical density of cells was measured at 620 nm by using a spectrometer (Model Gnesys 10 Bio, Thermo Scientific, United States). For dry cell weight determination, 10 ml of samples were centrifuged and pellets were left drying at 37°C to constant weight. A calibration curve was also constructed to relate OD_{620} values and cell dry weigh (Brinques *et al*., 2010; Cutting, 2011).

Residual sugar content of the cultivation medium was determined by using 3,5-dinitrosalicylic acid (DNS) method (Brinques *et al*., 2010; Cutting, 2011; Das *et al*., 2010). A 0.1 ml of each sample was mixed with 3.9 ml of distilled water and 0.08 ml of HCl in a glass tube for hydrolysis of sugars. The solution was mixed and then heated in a water bath at 90°C. After neutralization with 0.2 ml of 5 N KOH, 3 ml of solution was transferred into a clean test tube and 3 ml of DNSA solution (10 g/L dinitrosalicylic acid, 0.5 g/L sodium sulfite and 10 g/L sodium hydroxide) was added to the solution. 3 ml of distilled water was also mixed with 3 ml DNSA solution to be used as blank in the spectrophotometric measurements. The solution heated in a water bath at 90°C for 10 min. A color change was observed during heat treatment and in order to stabilize the color in the solution, 1 ml of 40% potassium sodium tartrate solution was added to each tube. The test tubes were mixed and cooled to room temperature in a water bath. Absorbance measurements were done at 575 nm and recorded. A standard curve was also constructed for each experimental run by using sterile cultivation medium. The medium was serially diluted and the same procedure of DNS method was performed.

Results and Discussion

In the first optimization step, in order to enhance biomass production of *B. pumilus* STF26, three variables; temperature, pH and carbon concentration were optimized by using response surface methodology. Temperatures in the range of 25°C to 40°C, pH from 5.0 to 8.0, and dextrose concentration from 5.0% to 20.0% (w/v) were analyzed. A full quadratic response surface model was constructed by using Minitab with coded units, and the following equation relating the biomass and the test variables was obtained:

$$Y\,(\text{biomass}) = 7.2267 - 1.4875x_1 + 1.1562x_2 + 0.4487x_3 - 2.6083x_1^2 - 1.5058x_2^2 + 0.6242x_3^2 - 0.5175x_1x_2 + 0.4725x_1x_3 - 0.4200x_2x_3 \quad (3)$$

where Y is the response value which is biomass, x_1, x_2 and x_3 are coded values of the factors tested which are temperature, pH and dextrose concentration respectively. The significance of the coefficients in the model was determined by p values Table 1.

Smaller magnitude of p values indicates higher significance of the corresponding coefficient (Barbosa *et al*., 2005). According to the present model, temperature, pH and quadratic effects of

Table 1. Response surface regression results for first optimization**

Term	Coefficient	Standard error of coefficient	t value	p value
Constant	7.2267	0.4831	14.959	0.000
x_1	-1.4875	0.2958	-5.028	0.004*
x_2	1.1562	0.2958	3.908	0.011*
x_3	0.4487	0.2958	1.517	0.190
x_1x_1	-2.6083	0.4355	-5.990	0.002*
x_2x_2	-1.5058	0.4355	-3.458	0.018*
x_3x_3	0.6242	0.4355	1.433	0.211
x_1x_2	-0.5175	0.4184	-1.237	0.271
x_1x_3	0.4725	0.4184	1.129	0.310
x_2x_3	-0.4200	0.4184	-1.004	0.362

$R^2 = 95.05\%$, R^2 (adj) $= 86.15\%$, $p_{(lack\ of\ fit)} = 0.38$
* $p < 0.05$ is significant.
** x_1, x_2 and x_3 represents temperature (°C), pH, and dextrose concentration (%, w/v) respectively.

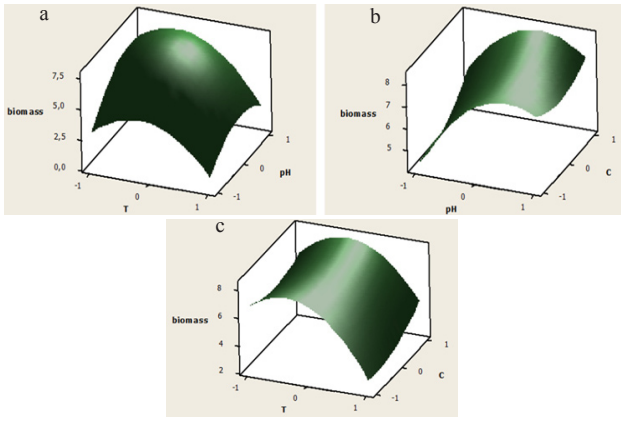

Figure 1. Response surface plots of (a) temperature and pH, (b) pH and carbon source concentration, and (c) temperature and carbon source concentration on biomass production of *Bacillus pumilus* STF26 by holding other factors constant at middle point of the Box-Behnken design.

them were significant for biomass production. In Figure 1, response surface plots of (a) temperature and pH, (b) pH and carbon source concentration, and (c) temperature and carbon source concentration on biomass production of *Bacillus pumilus* STF26 by holding other factors constant at middle point of the Box-Behnken design was shown. The optimum values for temperature, pH and carbon source concentration were found as 30.9°C, 6.9 and 20% (w/v), respectively.

In order to verify the optimum values of the variables obtained by RSM, an experiment was conducted with the optimum values of the test variables and the maximum biomass was obtained as 8.35 g/L, very close to the predicted value 8.52 g/L. Other medium components were constant at the concentrations of yeast extract, 20 g/L; KH_2PO_4, 2 g/L and $MgSO_4.7H_2O$, 1 g/L. Agitation speed and air flow rate were also fixed at 200 rpm and 2 vvm, respectively. Maximum biomass concentration of 8.35 g/L was obtained at 22 h, beginning of the stationary phase.

Although results show that the maximum biomass was obtained at the highest dextrose concentration, according to the results of DNS assay all of the sugar in the cultivation medium was not consumed.

Table 2. Box-Behnken design matrix of the second optimization with three variables in coded and uncoded units and with the response, biomass

Trial No:	2nd optimization x_4	x_5	x_6	Biomass (g/L) Exp	Estimated
1	0.2 (-1)	0.1 (-1)	0.26 (0)	2.89	2.83
2	2 (+1)	0.1 (-1)	0.26 (0)	5.62	5.52
3	0.2 (-1)	0.5 (+1)	0.26 (0)	2.85	2.71
4	2 (+1)	0.5 (+1)	0.26 (0)	4.93	4.91
5	0.2 (-1)	0.3 (0)	0.02 (-1)	2.52	2.55
6	2 (+1)	0.3 (0)	0.02 (-1)	5.68	5.71
7	0.2 (-1)	0.3 (0)	0.5 (+1)	2.44	2.41
8	2 (+1)	0.3 (0)	0.5 (+1)	7.35	7.12
9	1.1 (0)	0.1 (-1)	0.02 (-1)	6.57	6.65
10	1.1 (0)	0.5 (+1)	0.02 (-1)	8.52	8.49
11	1.1 (0)	0.1 (-1)	0.5 (+1)	9.69	9.72
12	1.1 (0)	0.5 (+1)	0.5 (+1)	5.09	5.12
13	1.1 (0)	0.3 (0)	0.26 (0)	5.41	5.42
14	1.1 (0)	0.3 (0)	0.26 (0)	5.54	5.51
15	1.1 (0)	0.3 (0)	0.26 (0)	5.34	5.53

*x_4 is yeast extract concentration (%, w/v), x_5 is KH_2PO_4 concentration (%, w/v) and x_6 is $MgSO_4.7H_2O$ concentration (%, w/v).

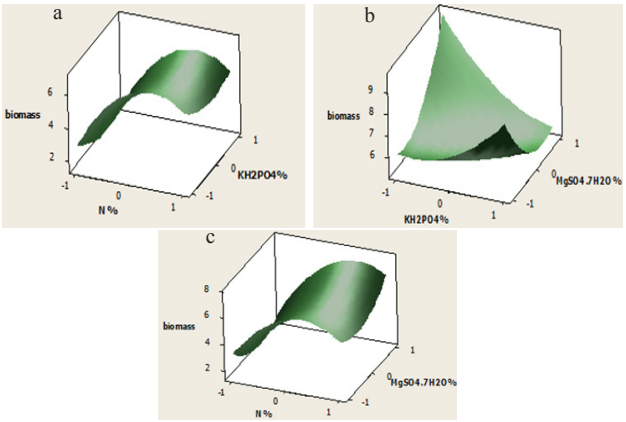

Figure 2. Response surface plots of (a) nitrogen source concentration and KH_2PO_4 concentration (b) KH_2PO_4 concentration and $MgSO_4.7H_2O$ concentration, and (c) nitrogen source concentration and carbon source concentration on biomass production of *Bacillus pumilus* STF26 by holding other factors constant at middle point of the Box-Behnken design.

When only the consumed amount of dextrose was put into the growth medium, biomass production decreased. The reason for this might be that while dextrose at high concentrations triggers the growth of the microorganism, the organism cannot consume it completely.

After optimizing temperature, pH and dextrose concentration, the concentrations of yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$ were optimized using response surface methodology. Concentrations of yeast extract in the range of 2 to 20 g/L, KH_2PO_4 from 1 to 5 g/L and $MgSO_4.7H_2O$ from 0.2 to 5 g/L were tested. Temperature, pH and dextrose concentration were set to the values obtained from first optimization as 30.9°C, 6.9 and 20% (w/v) respectively. Test variables with coded and uncoded units and the response values are given in Table 2. Regression analysis and the following equation were obtained that relates biomass and the factors tested:

$$Y\ (biomass) = 5.4300 + 1.6100x_4 - 0.4225x_5 + 0.1600x_6 - 2.1638x_4^2 + 0.8063x_5^2 + 1.2312x_6^2 0.1625x_4x_5 + 0.4375x_4x_6 - 1.6375x_5x_6 \quad (4)$$

Table 3. Response surface regression results for first optimization**

Term	Coefficient	Standard error of coefficient	t value	*p* value
Constant	5.4300	0.3883	13.986	0.000
x_4	1.6100	0.2378	6.772	0.001*
x_5	-0.4225	0.2378	-1.777	0.136
x_6	0.1600	0.2378	0.673	0.531
x_4-x_4	-2.2638	0.3500	-6.183	0.002*
x_5-x_5	0.8063	0.3500	2.304	0.069
x_6-x_6	1.2312	0.3500	3.518	0.017*
x_4-x_5	-0.1625	0.3362	-0.483	0.649
x_4-x_6	0.4375	0.3362	1.301	0.250
x_5-x_6	-1.6375	0.3362	-4.870	0.005*

$R^2 = 96.45\,\%$, $R^2\,(\text{adj}) = 90.05\,\%$, $p_{(\text{lack of fit})} = 0.14$
* $p < 0.05$ is significant.
** x_4, x_5 and x_6 represents the concentrations of yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$ respectively.

where Y is the biomass concentration, x_4, x_5 and x_6 are coded values of the concentrations of yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$, respectively.

Table 3 shows the regression coefficients of the 2nd optimization model and the p values. According to the p values of the present model, concentration of yeast extract, quadratic effects of yeast extract concentration and $MgSO_4.7H_2O$ concentration and the interaction of KH_2PO_4 and $MgSO_4.7H_2O$ concentrations have significant effects on the biomass production. Although other coefficients in the model do not affect significantly on biomass, all terms were included in Equation (4) since the R^2 value, 0.96, was showing that the model was very reliable.

Response surface was constructed for the second optimization in order to observe the effects of interactions between two factors tested Figure 2. The elliptical shape of the response surface showing the interaction between KH_2PO_4 and $MgSO_4.7H_2O$ indicate that this interaction has significant effect on biomass production of *B. pumilus* STF26. The optimum values were found as X_4 = 1.526% (w/v), X_5 = 0.1% (w/v) and X_6 = 0.5% (w/v).

Optimization results were confirmed by conducting an experiment with the optimum values of the test variables obtained by response surface methodology. Maximum biomass was measured as 10.42 g/L which is close to the predicted value (10.17 g/L) found by the optimization of the regression equation (Equation (4)). Other variables (temperature, pH and dextrose concentration) were constant at their optimum values that were found out in first optimization. Agitation speed and air flow rate were again set to 200 rpm and 2 vvm respectively. Maximum biomass concentration was obtained as 10.42 g/L at 24 h, beginning of the stationary phase.

Although results show that the maximum biomass was obtained at the highest dextrose concentration, according to the results of DNS assay all of the sugar in the cultivation medium was not consumed. When only the consumed amount of dextrose was put into the growth medium, biomass production decreased. The reason for this might be that while dextrose at high concentrations triggers the growth of the microorganism, the organism cannot consume it completely. After two steps of optimization, it is determined that optimum concentrations of the medium components were 20% dextrose (w/v), 1.526% yeast extract (w/v), 0.1% KH_2PO_4 (w/v) and 0.5% $MgSO_4.7H_2O$ (w/v) to obtain maximum concentration of *B. pumilus* STF26 biomass and optimum cultivation conditions were 30.9°C and 6.9 pH.

Finding out the optimum concentrations of the medium components, growth of STF26 in optimized medium was compared with the one in LB medium. Other cultivation conditions were the same in both media where temperature and pH were at their optimized values. Maximum biomass concentration obtained when the culture was grown in LB was 4.23 g/L, nearly 2.5 times lower than the value obtained when the culture was grown in optimized medium.

Conclusions

Optimization of the cultivation conditions and the medium composition are of crucial importance since they considerably affect overall process economics. In this study, in order to maximize the biomass of a potential probiotic strain, concentrations of four main medium components (dextrose, yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$), temperature and the pH values were optimized by using response surface methodology for this commercially important microorganism. RSM is a more advantageous technique than the conventional one-factor-at-a-time method, since it is less time-consuming and it also analyzes the interactive effects among the variables tested. The results demonstrate that optimum values of temperature, pH, dextrose concentration, yeast extract concentration, KH_2PO_4 concentration and $MgSO_4.7H_2O$ concentration are 30.9°C, 6.9, 20% (w/v), 1.526% (w/v), 0.1% (w/v) and 0.5% (w/v) respectively to obtain maximum biomass. Maximum biomass obtained at optimized conditions was 10.42 g/L and this value was considerably higher when it was compared with the value obtained by using LB medium. After second optimization studies, first optimization can be repeated by using the optimized values of yeast extract concentration, KH_2PO_4 concentration and $MgSO_4.7H_2O$ concentration in order to check the goodness of the optimum temperature, pH and dextrose concentration values. Biomass of this microorganism can be further increased by optimizing other cultivation conditions

such as air flow rate and agitation speed.

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