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Production Of 2, 5-Hexanediol Using Pichia farinose: A Response Surface Methodology

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Abstract: Hexanediol is being used in perfume, medicine and as an intermediate in the preparation of optically active tetrahydrofurans used in biodegradable polymers. Commercially hexanediol is produced chemically which is a very cost effective with very low productivity. In this project we have tried to produce Hexanediol using *Pichia farinose* MTCC*246 (a yeast) and have optimized the process for batch production. Box-Beckmen method of Response Surface Methodology (Minitab14® Software) was used for the optimization of temperature, pH, glucose and hexanedione concentration (substrate). The final product Hexanediol in fermentation broth was extracted using ethyl acetate and estimated using Gas chromatography (Perkin Elmer USA). The optimum temperature, pH, glucose, substrate concentration and time for the growth of micro-organism and Hexanediol production were found to be 33.038˚C, 7.53, 55g/l, 5.5mM and 48hrs respectively.
Key words: Hexanediol, optimum conditions, hexanedione, *Pichia farinose*, Box-Beckmen method of Response Surface Methodology.

1. Introduction

Microbial transformations or yeast-mediated transformations for the production of various economically important products and compounds have been widely used since the early days of mankind, for example, the production of bread, dairy products, and alcoholic beverages. Hexanediol is a versatile building block for the synthesis of various chiral phosphine ligands, which are used in chiral Wilkinson catalysts[1,2,3]. Also hexanediol is used as intermediate in the preparation of optically active tetrahydrofurans used in biodegradable polymers, perfumes and medicines. The established industrial production process for hexanediol is a multistep synthesis starting with an enantioselective acylation of the (R)-hydroxy function of the racemic/meso hexanediol mixture catalyzed by a lipase[4,5]. Subsequently, the nonacylated (S)-hydroxy function of the meso-(R,S)-diol is inverted by chemical transformation with methane sulfonyl chloride leading to the (R,R)-diol. Reagents used in the

production process are triethylamine, methane sulfonyl chloride, dichloromethane, dimethyl formamide, cesium acetate, methanol, and acidic resins (Amberlite IR 120). The maximum theoretical yield of this process is 75%[6]. However, no information has been published on the real yields achieved in this process. So the industrial production of hexanediol is very expensive. Microbial approach using resting whole cells to produce hexanediol was published by Hummel et al in 2002. Lactobacillus kefir used to reduce 2,5-hexanedione to 2,5-hexanediol[7]. But the product concentration in this process is very low and many problems arise with morphology of the cells. Moreover the bacterial cell cannot withstand high concentrations of substrate. It causes substrate inhibition. It was found that the enzyme used for the biotransformation of 2,5-hexanedione to 2,5-hexanediol was found to be alcohol dehydrogenase(ADH). This is a secondary metabolite. The production of this enzyme is triggered by growing the microorganism at anaerobic conditions.

Response surface methodology (RSM) explores the relationships between several explanatory variables and one or more response variables. The method was introduced by G. E. P. Box and K.B. Wilson in 1951. The main idea of RSM is to use a set of designed experiments to obtain an optimal response. Experimental designs used in RSM must make tradeoffs between reducing variability and reducing the negative impact that can be caused by bias. The Box-Behnken design for 3 factors involves three blocks, in each of which 2 factors are varied through the 4 possible combinations of high and low[8]. It is necessary to include centre points as well (in which all factors are at their central values). In this study we report the growth behavior and 2,5-hexanediol production by *Pichia farinosa*. The experiments were designed using Box-Behnken a Response Surface Methodology (RSM) in Minitab 15® software and the results were optimized.

2. Materials and methods

2.1. Source of Microorganism

Pichia farinose (MTCC*246) was obtained from the Microbial Type Culture Collection and gene bank, Chandigarh, India was maintained in Malt yeast agar medium (MY agar, HImedia India)

2.2. Pre-culturing Conditions

A preculture was prepared by transforming a loopful of microorganisms grown on YM agar slants at 30°C. After 48h the sporulated cultures were stored at 4°C for no longer than 1 month, the stock culture of the strain was maintained by periodic sub-culture on the same nutrient medium.

2.3. Growth Media and Culture Conditions

Pichia farinose (MTCC *246) culture was grown in 100 ml flask containing the following medium: Glucose 20(g/l), Peptone 7(g/l), Yeast extract 5(g/l), K_2HPO_4 2(g/l) and KH_2PO_4 3(g/l). The medium was sterilized at 121°C for 20 minutes before inoculation. This was maintained at aerobic conditions. Then the different media were prepared based on the runs created using Minitab 15®. Inoculation was performed using 5% (v/v) of yeast suspension. These cultures were maintained at 80rpm and maintained at anaerobic conditions. The production of 2.5-hexanediol depends on the production of alcohol dehydrogenase. This is a secondary metabolite. Its production is induced at anaerobic conditions.

2.4. Analytical Techniques

2.4.1 Estimation of cell growth

The dry weight was measured by harvesting culture at room temperature. 10ml of the culture was taken and centrifuged at 10,000 rpm. The pellet was dried at 70°C for 2 hours. Then the dry weights of the cultures were measured.

2.4.2 Standardization of 2,5-Hexanedione

Ten samples of different concentrations of 2,5-hexanedione was prepared(1mM to 10mM). To 10ml of all the samples 2ml of Ehrlich's reagent was added. O.D was measured at 525nm. The standard graph was plotted.

2.4.3 Estimation of 2,5-Hexaedione concentration

The estimation of 2,5-Hexanedione was carried out by spectrophotometric methods[9]. The enzyme produced by the yeast species was found to be extra cellular. Hence after centrifugation the supernatant was taken for analysis. 5ml of an ethyl acetate extract of the supernatant was prepared, and to this 2 ml of Ehrlich's reagent

was added. This formed a light yellowish green liquid. Its absorbance was measured at 525 nm. The concentration of 2,5-Hexanediol was found by comparing the O.D with the standard.

2.5. Experimental design and statistical analysis

The variables pH, (X1), Temperature (X2), Glucose concentration (X3), Hexanedione concentration (X4) and Time (X5) were explored, each at three levels in both coded and actual units **(Table 1).** The Box-Behnken (BB) experimental design was developed as given in Table 2 using MINITAB 15 software. Based on the regression analysis following second order polynomial model (equation 1) describing the relationship between the independent variables and L-asparaginase activity was developed.

$$y = \beta_0 + \sum_{1}^{k}\beta_i x_i + \sum_{1}^{k}\beta_{ii} x_i^2 + \sum_{i<j}\sum\beta_{ij} x_i x_j + \varepsilon \quad \ldots\ldots(1)$$

where Y is the predicted response, k the number of factor variables, β0 the model constant, βi the linear coefficient, Xi the factor variable in its coded form, βii the quadratic coefficient, and βij is the interaction coefficient[8]. All experimental designs were randomized to exclude any bias, experiments were performed in duplicate and the averages of the observations were used. The polynomial model was validated using F-test for analysis of variance (ANOVA) and optimized for optimal values of the variables[10,11,12].

Table- 1 Codes and actual levels of independent variables for design of experiment

Independent Variables	Coded levels		
	-1	0	1
pH	6	7	8
Temperature (°C)	25	35	40
Time (hours)	36	42	48
Glucose conc., (gram)	2.5	4	5.5
Hexanedione conc., (mM)	4.5	5	5.5

Table 2 Central composite design in coded units of the variables

Run Order	pH	Temp (°C)	Glucose (mg/100ml)	Time (hrs)	Hexanedione (mM)	Hexanedione consumed(mM)
1	8	25.0	4.0	42	5.0	2.35
2	8	32.5	4.0	42	4.5	2.17
3	6	32.5	5.5	42	5.0	3.49
4	7	32.5	2.5	36	5.0	0.89
5	7	25.0	4.0	42	5.5	2.45
6	6	32.5	4.0	48	5.0	2.24
7	7	32.5	5.5	48	5.0	0.97
8	7	32.5	4.0	36	4.5	1.58
9	8	32.5	4.0	36	5.0	3.58
10	8	40.0	4.0	42	5.0	2.12
11	7	32.5	4.0	42	5.0	1.40
12	7	32.5	5.5	42	5.5	1.05
13	6	32.5	2.5	42	5.0	1.38
14	7	32.5	2.5	48	5.0	1.01
15	7	25.0	5.5	42	5.0	2.45
16	7	32.5	4.0	42	5.0	1.26
17	8	32.5	4.0	48	5.0	2.01
18	7	40.0	4.0	48	5.0	3.16
19	8	32.5	4.0	42	5.5	1.97
20	7	40.0	4.0	42	4.5	3.24

21	7	32.5	5.5	42	4.5	0.78
22	7	32.5	4.0	36	5.5	2.05
23	8	32.5	2.5	42	5.0	4.00
24	7	32.5	2.5	42	5.5	2.83
25	7	25.0	4.0	42	4.5	2.41
26	6	32.5	4.0	42	4.5	2.93
27	7	25.0	4.0	36	5.0	2.90
28	6	32.5	4.0	42	5.5	3.13
29	7	40.0	2.5	42	5.0	4.30
30	6	25.0	4.0	42	5.0	4.20
31	7	32.5	2.5	42	4.5	3.46
32	7	32.5	4.0	42	5.0	2.00
33	7	32.5	4.0	48	4.5	1.80
34	7	25.0	2.5	42	5.0	2.50
35	7	32.5	4.0	42	5.0	0.54
36	7	40.0	4.0	36	5.0	2.98
37	7	32.5	4.0	48	5.5	0.72
38	8	32.5	5.5	42	5.0	2.70
39	6	40.0	4.0	42	5.0	3.45
40	7	25.0	4.0	48	5.0	3.20
41	6	32.5	4.0	36	5.0	3.51
42	7	40.0	5.5	42	5.0	2.78
43	7	32.5	4.0	42	5.0	1.02
44	7	32.5	5.5	36	5.0	2.50
45	7	32.5	4.0	42	5.0	0.52
46	7	40.0	4.0	42	5.5	3.00

3. Results and discussion

All the 46 runs were conducted, and the results tabulated (Table 2), the optimization was done using Minitab15®. For a better understanding about the interactions among the variables on the response the surface plot was also plotted. The 3D response surfaces based on independent variables were obtained using the same software package (Fig 1 to 10) indicated that a local optimum exists in the area experimentally investigated. The orientation of the principal axes of the contour plots between the variables pH and temperature, pH and fermentation time indicated that the mutual interactions between these set of variables had a significant effect on the product yield. When the third independent variable fermentation time was kept constant at 48 h, glucose concentration at 55mg/l (Fig 7) the interaction between the two variables (temperature and pH) showed that the product yield was sensitive even when pH and temperature were subject to small alterations. Under certain condition a maximal contour could be determined, meaning that further change in temperature and pH would not increase the product yield any further. The results showed that as the values of process variables increased, the yield also increased but only up to the midpoint of range of variables and thereafter the yield decreased even though the values of variables increased. The product yield was significantly affected by pH, temperature, glucose, substrate concentration and fermentation time where pH producing greater effect. The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables, and effects of interactions of each independent variable, on the response variables. The shape of the corresponding 3D surface plots indicates whether the mutual interactions between the independent variables are significant or not. From the 3D response surface plots and the corresponding contour plots, the optimal values of the independent variables could be observed, and the interaction between each independent variable pair can de described. Based on model, the optimal working conditions were obtained to attain high product yield.

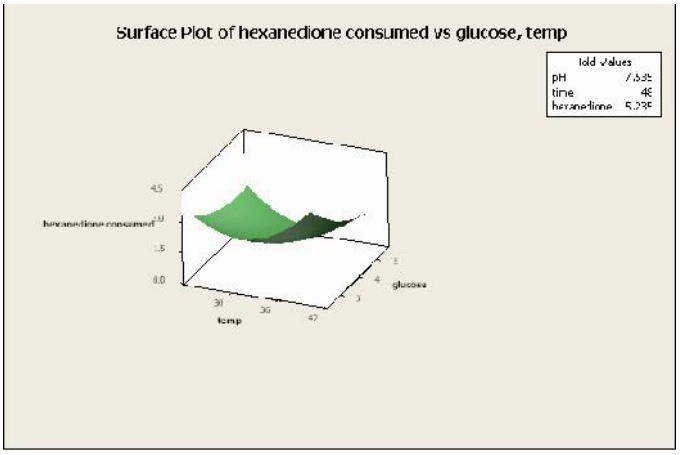

Fig. 1. Surface plot for effect of temperature and glucose on Hexanedione Consumption

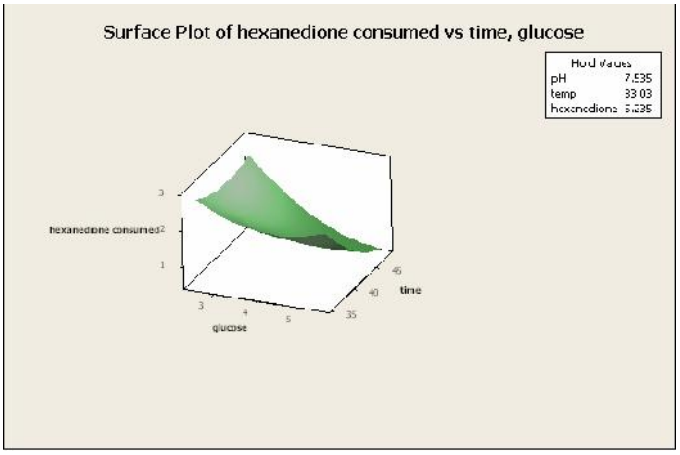

Fig. 2. Surface plot for effect of time and glucose on Hexanedione consumption

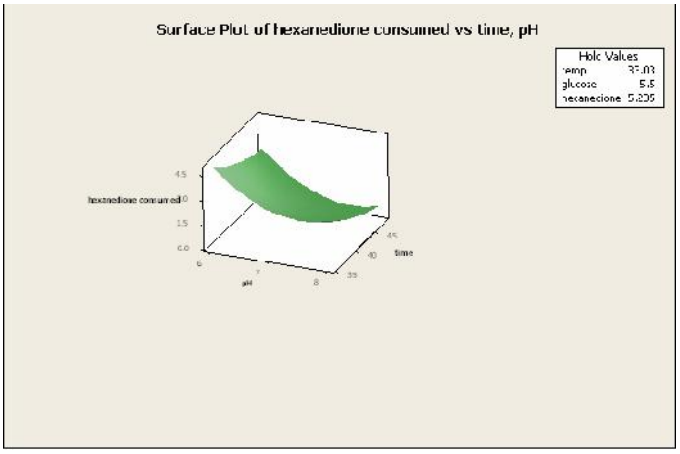

Fig. 3. Surface plot for effect of pH and time on Hexanedione consumption

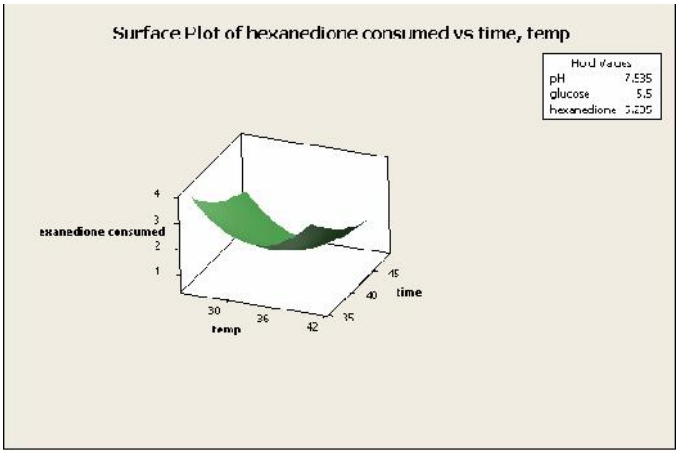

Fig. 4. Surface plot for effect of temperature and time on Hexanedione consumption

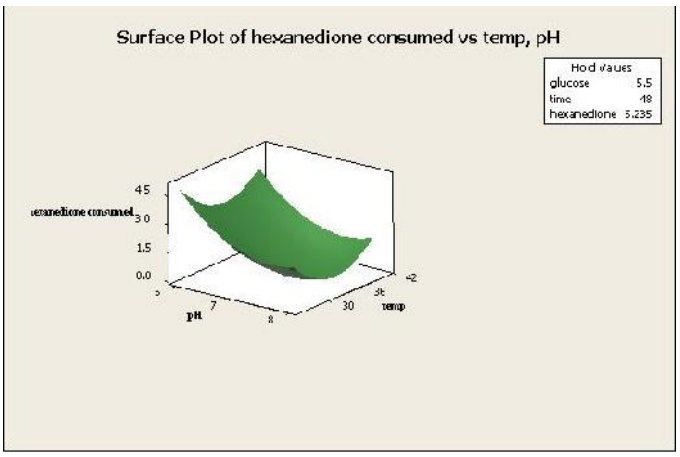

Fig. 5. Surface plot for effect of pH and temperature on Hexanedione consumption

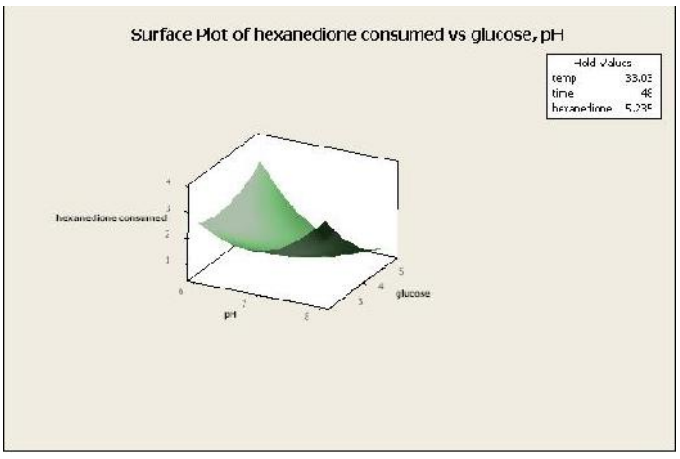

Fig. 6. Surface plot for effect of pH and glucose on Hexanedione consumption

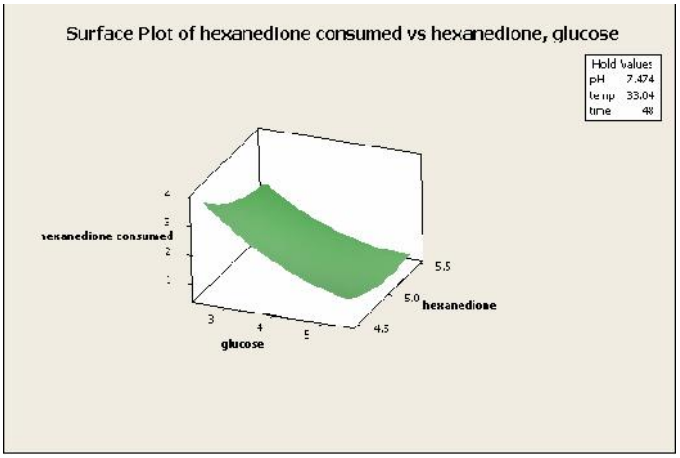

Fig. 7. Surface plot for effect of Hexanedione and glucose on Hexanedione consumption

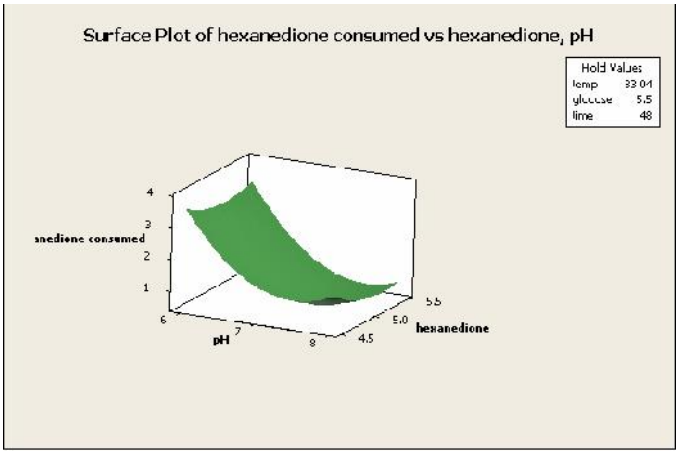

Fig. 8. Surface plot for effect of Hexanedione and pH on Hexanedione consumption

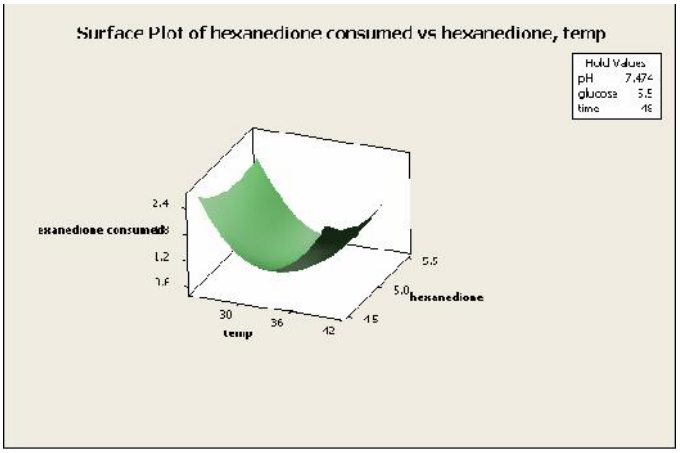

Fig. 9. Surface plot for effect of Hexanedione and temperature on Hexanedione consumption

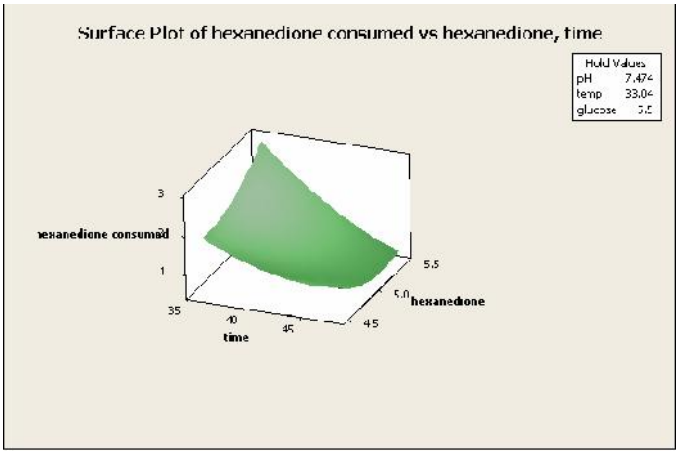

Fig. 10. Surface plot for effect of Hexanedione and time on Hexanedione consumption

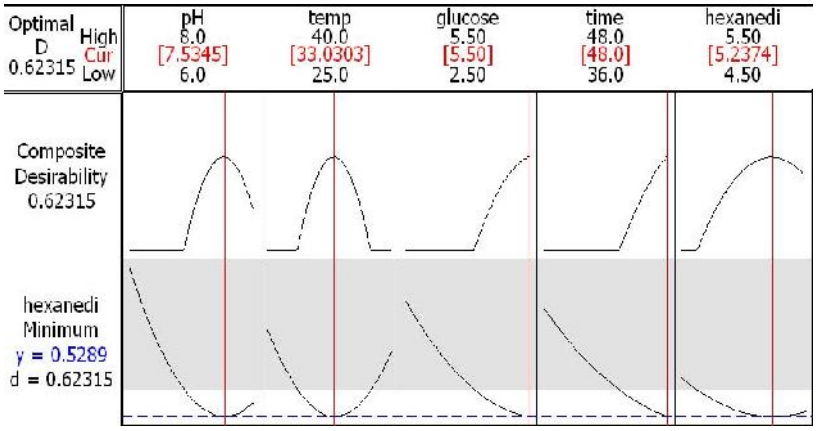

Fig. 11. Optimization plot for Hexanedione consumption

4. Conclusions

The production of Hexanediol using *Pichia farinose* MTCC *246 (a yeast) was studied and optimized the process for batch production. Box- Behnken method of Response Surface Methodology (Minitab14® Software) was used for the optimization of temperature, pH, glucose and hexanedione concentration (substrate).The values of the optimized variables were found to be pH 7.53, Temperature 33.03oC, Glucose conc., 5.5 mg/100ml, Time 48hrs, Hexanediol conc., 5.23mM.

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