OPTIMIZING PROCESS PARAMETERS OF ETHANOL PRODUCTION USING SACCHAROMYCES CEREVISIAE MTCC 178

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Abstract: Optimization is the process of obtaining the most suitable results under the given set of conditions for an experiment. It finds its application in all the fields of Engineering. While designing any product or process, the engineers and scientists have to obtain a point where all the influencing parameters combine to give the most desirable results in the best possible way. Response Surface Methodology is a powerful technique which performs optimization based on experimental designs in order to maximize their performance. In a multifactor complex system, the interaction of the various factors is studied effectively by a full factorial design. This study is to provide an insight into the application of the statistical tool, Response Surface Methodology in the optimization of biological processes and also summarizes a case study of using Central Composite Design for optimization of a fermentation process of ethanol production by *Saccharomyces cerevisiae* MTCC 178 strain using sorghum juice as the media with varying pH, nitrogen and phosphorous concentrations.

Keywords Ethanol Production, MTCC 178 strain of *Saccharomyces cerevisae,* Process Optimisation, Response Surface Methodology, Sorghum Juice

Introduction: Optimization is the procedure of obtaining the most appropriate solution for a problem among the various constraints present. Obtaining the solution to an optimization problem consists of few steps i.e. describing the problem in detail, formulation of a suitable mathematical model, verification of the model and finally its implementation (Nagesh Kumar, 2012). One of the most powerful response optimization statistical technique is Response Surface Methodology (John M. Simbala, 2009).It is extensively used because it involves the creation of a response surface which makes the optimization calculations easier.RSM is used to establish a functional relationship between the input variables and the response variables of an experiment. Two important models of RSM for a response variable,y and input variables ($x_1,x_2......x_k$),Є a random experimental error, assumed to have zero mean, βi, βj, βij coefficients estimated from regression, are the first degree model

$$y=\beta_\circ+\sum^{k}_{i=1}\beta_i x_i+\epsilon \quad (1)$$

and the second degree model

$$y=\beta_\circ+\sum^{k}_{i=1}\beta_i x_i+\sum_{i<j}\sum\beta_{ij}x_i x_j+\sum^{k}_{i=1}\beta_{ii}x_i^2+\epsilon$$

(Khuri,2006) (2)

Box and Wilson gave RSM in 1951 and involves fitting first and second order models into response surface designs and then validating the adequacy of the model by statistical analysis (Akpan et al.,2013).

RSM has varied applications such as evaluating a scientific activity (Evaristo et al.,2009),finding optimum process parameters for extraction of a fruit pulp(Bafna,2012),optimization of paper Nanocoating (Diaconescu et. Al,2011),studying contribution of parameters such as cutting speed and feed size, surface integrity measured in terms of microhardness etc. to the milling of a cantilever shaped plate(Bhopale & Pawade,2012) and so on.

Response Surface Methodology is widely used in studying process parameters in many biological processes especially response surface design, Central Composite Design, for fermentation studies(Fung et. al.,2013), viz. Optimizing process parameters of enzyme production by fungi (Vimalashanmugam & Viruthagiri, 2012) observing effect of parameters such as acetic acid,pH and ethanol on intracellular pH of fermenting yeast (Pampulha & Loureiro-Dias,1989),Optimizing ethanol production from various biological sources namely lignocellulosic biomass (Han et. al.,2011) ,Tapoica starch oil (Manikandan & Viruthagiri, 2010) ,bagasse pith hydrolysate (Dasgupta et. al., 2013),corn flour (Manikandan & Viruthagiri, 2010),palm trunk sap (Norhazimah et. al., 2012) ,finger millet medium (Puligundla et.al., 2012) etc.

The ever increasing demand of fuel and the depletion of non-renewable energy sources has led to the need of finding alternative sources of energy such as bioethanol. Use of fossil fuels resulted in problems of global warming (Archer, 2005) however using agricultural crops or plants for ethanol production and eventually using it as fuel is a renewable fuel used for road transport application (Sanchez,2007).Sorghum is a fast growing crop (Mutepe et. al., 2011) and considered a promising crop for ethanol production at low cost (Barbanti et. al., 2006).

The objective of this paper is to show the use of Response Surface Methodology, an optimization technique, for optimizing parameters of biological processes by demonstrating a study of effect of parameters namely pH,nitrogen concentration(supplemented by $(NH_4)_2SO_4$) and phosphorous concentration(supplemented by KH_2PO_4) in the sorghum juice used as media for ethanol production by fermentation using *Saccharomyces cerevisiae* MTCC 178 strain.

Methods:

Revival of yeast strain: *Saccharomyces cerevisiae* MTCC 178 strain was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Yeast strain MTCC 178 was initially stored in freeze dried form in an ampoule in -4°C freezer. After procuring sample from the freezer, the content from the ampoule was poured into test tubes with 1.8 cm^3 physiological solution. In order to activate the yeast cells, the test tube was put in a thermostat at 30°C for 30 minutes – time sufficient to restore their viability. After restoring the viability of yeast next step is to perform culturing and sub culturing on media plates.

Sub-culturing of yeast: Preparation of media plates:

Yeast extract - 1 g/100ml
Dextrose - 2 g/100ml
Peptone - 2 g/100ml
Agar - 2 g/100ml

All the chemicals were weighed individually and put into the 200 ml flask. After that volume was made up to 100ml by using distilled water. The growth media was then autoclaved at 121°C and 15 psi. The autoclaved media then poured into Petri-plates in the laminar flow and allowed them to gel. After preparation of media plates, these were then inoculated with the yeast strain (revived in the above method) through streaking. After plating, plates were then sealed using parafilm and then kept in the incubator (30°C) for 24 hours. After 24 hours some of the cells from the previous culture are transferred to a new petri-plate using inoculation loop. This is called sub-culturing it is done to prolong life and increase the number of cell of microorganisms. Sub-culturing was performed thrice.

Preparation of Cellulase enzyme stock: Cellulase enzyme is present in powder form so prior to use it is first dissolved in phosphate buffer. As cellulase works best at at pH 4.8 so the pH of the phosphate buffer was set by mixing monobasic and dibasic in a particular ratio.

Monobasic (KH_2PO_4) – 136.825 g/l
Dibasic (K_2HPO_4) – 2.283g/l

Sorghum syrup preparation: Fresh Sorghum stalks were collected from fields. 100gm of sorghum stalks were taken and using gloves and sterilized blades were peeled off. They were later washed repeatedly with tap water followed by distilled water. The stem pieces were crushed in a blender and processed with a dilution of approximately 1:1(using 100ml of double distilled water) to obtain a slurry. The slurry was mixed with cellulase enzyme with pH 4.8 and left for overnight incubation at 37°C.The enzymatic hydrolysis was followed by filtering the slurry using a two layered muslin cloth and a translucent solution was obtained. The left over impurities were removed by centrifuging the juice at around 5500 rpm and 4°C for 10 minutes.The supernatant was carefully collected in an autoclaved glass beaker leaving the pellet behind. The beaker was covered with aluminum foil and stored at 4°C.

Production of alcohol from sorghum juice: Sorghum juice was used as a substrate for alcohol production. Through response surface methodology (design expert software) run sheet was prepared. A run sheet containing pH values (1.64,3,5,7,8.36),nitrogen concentration(0.99,1.40,2.00,2.60,3.01) and phosphorous concentration values (0.10,1.35,2.60,3.45)in different combination was obtained. Before initiating the fermentation process, a YEPD broth of 100 ml was prepared and autoclaved. A single colony of yeast strain was picked from the petriplate and was added into the YEPD broth and was kept in the incubator-shaker (30°C, 200rpm) for 8-9 hours.

The fermentation was carried out in 50 ml falcon tubes. All the falcon tubes were labeled with different combination of conditions according to the data procured from the software.10 ml of sorghum juice was poured in each sterilized falcon tube and the pH was set individually in all falcon tubes according to the marked conditions using 1N NaOH and 1N HCl. After that the yeast strain was added into the falcon tubes in an amount 5% of the total volume present in each test tube after the maintenance of pH. After adding yeast sample in all the falcon tubes they were then kept in incubator-shakers at 37°C, 200rpm. The alcohol concentration and brix was measured through Refractrometer from 1-7 days and the readings were carefully noted.

Table 1: Experimental set-ups generated through design expert software and the corresponding responses obtained by experiments

Select	Std	Run	Factor 1 A:pH	Factor 2 B:N g/l	Factor 3 C:P g/l	Response 1 alcohol %	Response 2 brix degree brix
	4	1	7.00	2.60	0.10	12.8	6.5
	5	2	3.00	1.40	2.60	10.6	8
	12	3	5.00	3.01	1.35	11.3	8.4
	14	4	5.00	2.00	3.45	10.6	9.1
	9	5	1.64	2.00	1.35	4.3	15.7
	17	6	5.00	2.00	1.35	11.7	8.1
	3	7	3.00	2.60	0.10	11	8.8
	10	8	8.36	2.00	1.35	7.2	11.4
	15	9	5.00	2.00	1.35	11.7	8.1
	6	10	7.00	1.40	2.60	11.2	8.4
	20	11	5.00	2.00	1.35	11.7	8.1
	11	12	5.00	0.99	1.35	11.6	8.5
	7	13	3.00	2.60	2.60	9.4	10.5
	2	14	7.00	1.40	0.10	12.3	7.2
	8	15	7.00	2.60	2.60	12.4	7
	1	16	3.00	1.40	0.10	11.4	8.2
	13	17	5.00	2.00	1.35	11.7	8.1
	19	18	5.00	2.00	1.35	11.7	8.1
	18	19	5.00	2.00	1.35	11.7	8.1
	16	20	5.00	2.00	1.35	11.7	8.1

Experimental Set up: In the experiment, the effect of three factors i.e. pH, nitrogen concentration (in g/l) and phosphorous concentration (in g/l) present in fermentation media was observed in order to obtain maximum alcohol production with minimum brix. The high and low range for the three factors(input variables) was decided as per the range in which most of the yeast strains perform optimum fermentation i.e. pH (low-3,high-7),nitrogen concentration (low-1.40g/l,high-2.60g/l) and phosphorous concentration (low -0.10g/l,high-2.60 g/l). Response surface methodology was used to obtain a functional relationship between the input variables (pH, nitrogen concentration and phosphorous concentration) and responses (ethanol concentration and brix) of the ethanol fermentation using central composite design. A Central Composite Design was developed resulting in 20 experiments for fitting a second-order polynomial equation representing the relationship between the entire range of possible combinations of process parameters considered for optimization. The coded values of the process parameters were determined by the following equation:-

$$X_i = \frac{X_i - X_o}{X_o}$$

The regression analysis was performed of the data obtained followed by estimation of the coefficient of the regression equation. ANOVA analysis, comprising of various statistical techniques, was performed to check the adequacy of the mathematical model based on the regression equations obtained. The quadratic model equation was observed for the goodness of fit. The aptness of fit was evaluated by the coefficient of determination R^2, and F-test that helped in establishing its statistical significance. Each term in the equation was estimated by the goodness of fit for its significance. Response Surfaces to help in establishing the effect of concerned input factors on maximizing ethanol productivity and minimizing the brix were drawn.

3. Result Analysis: ANOVA (analysis of variance) was used to analyze the adequacy of the quadratic model for alcohol concentration and brix in table 2 and table 3 respectively

TABLE 2:-Analysis of variance table for the response surface quadratic model of alcohol concentration (response 1)

Source	Sum Of Squares	Degree Of Freedom	Mean Square	F Value	p-Value Prob>F	
Model	63.32	9	7.04	7.28	0.0023	Significant
A-pH	9.15	1	9.15	9.47	0.0117	
B-N	0.012	1	0.012	0.012	0.9135	
C-P	6.28	1	6.28	6.50	0.0289	
AB	1.36	1	1.36	1.41	0.2627	
AC	0.10	1	0.10	0.10	0.7528	
BC	1.250E-003	1	1.205E-003	1.294E-003	0.9720	
A^2	45.93	1	45.93	47.54	<0.0001	
B^2	0.69	1	0.69	0.72	0.4163	
C^2	4.37	1	4.37	4.52	0.0594	
Residual	9.66	10	0.97			
Lack of Fit	9.66	4	2.42			
Pure Error	0.000	6	0.000			
Corr.Total	72.98	19				

TABLE 3:-Analysis of variance table for the response surface quadratic model of brix(response 2)

Source	Sum Of Squares	Degree Of Freedom	Mean Square	F Value	p-Value Prob>F	
Model	1.41	9	0.16	5.07	0.0091	Significant
A-pH	0.33	1	0.33	10.51	0.0088	
B-N	7.269E-004	1	7.269E-004	0.023	0.8812	
C-P	0.15	1	0.15	4.85	0.0502	
AB	0.10	1	0.10	3.28	0.1002	
AC	6.567E-004	1	6.567E-004	0.021	0.8871	
BC	4.526E-003	1	4.526E-003	0.15	0.7102	
A^2	0.80	1	0.80	25.97	0.0005	
B^2	0.015	1	0.015	0.48	0.5055	
C^2	0.13	1	0.13	4.11	0.0702	
Residual	0.31	10	0.031			
Lack of Fit	0.31	4	0.007			
Pure Error	0.000	6	0.000			
Corr. Total	1.72	19				

The ANOVA of the quadratic models for both the responses indicate the models to be significant. The Model F-value of 7.28 and 5.07 respectively implied the model to be significant. There is only a 7.28% and 5.07% chances that a "Model F-Value" this large could occur due to noise. Model P value (Prob>F) are very low i.e. 0.0023 and 0.0091 for both the responses. This confirms that the models are significant. The F value and the corresponding P values, shown in table 2 and table 3 for all the input factors involved are essential to understand the mutual interactions between these variables.The terms of the model are significant if values of P are less than 0.0500 and thus the variables A(pH) and A^2(pH*pH) of both the models are significant. Adequate precision measures the signal to noise ratio and a ratio greater than 4 in value is considered to be desirable.It was found to be 10.835 and 7.638 respevtively and hence these models could be used to navigate the design space.

The R-squared,adjusted R squared and predicted R-squared values for both the response models for alcohol concentration and brix are (0.8676, 0.7485, -0.2921) and (0.8202,0.6584,-0.7898). The R-squared value closer to 1, indicates that the model being reliable for response prediction.

The mathematical models for the responses, alcohol concentration and brix are represented by the following second-order equations obtained by multiple regression analysis using the Design Expert Software 8.0.7 :-

Alcohol concentration=$+7.58875+3.82592(pH)-5.62642(N)-0.94875(P)+0.34375(pH*N)+0.045000(pH*P)-0.016667(N*P)-0.41650(pH^2)+0.97020(N^2)+0.15565(P^2)$.

Also the equation for the second response brix is:-

Brix=$+10.08764 -3.05814(pH) +6.44611(N) +0.35183(P)-0.54167(pH*N)+0.010000(pH*P)+0.20000(N*P)+0.36289(pH^2)-0.97657(N^2)-0.19106(P^2)$

In numerical optimization a goal was set for each factor as well as response. The goal set for each factor was in range and the goal set for the response 1 was maximized as we wanted the alcohol concentration in our juice to be maximum however that for response 2 i.e brix was minimized since we wanted brix to be minimum. The goals for factors and response were then combined into an overall desirability function. Desirability is an objective function that ranges from zero outside of the limits to one at the goal. The numerical optimization finds a point that maximizes the desirability function.

After setting up the criteria a solution sheet was generated showing the best response generated in function with other factors. According to the solution sheet generated ,the best solution was predicted to be observed in an optimized media having pH (5.83-5.93),Nitrogen Concentration(2.60g/l) and Phosphorous Concentration(0.10g/l) to obtain maximum alcohol concentration of around 12.8% and minimum brix i.e. 6.6 degree brix .

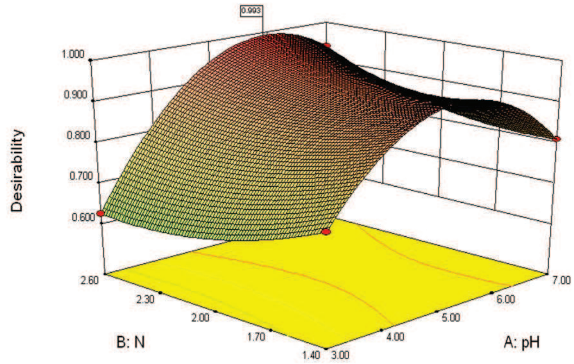

Fig :Numerical Optimization graph with Desirability representing the objective function of achieving the respective goals of the factors as well as responses.

Analyzing the mathematical correlation, it was observed that there was not much influence of adding mineral elements i.e nitrogen and phosphorous on the ethanol productivity.However pH was found to have a strong effect on the alcohol production by a yeast strain and was optimized well by the Response Surface Methodology.

Conclusion: Optimization is being done to obtain the most desirable results at minimum efforts. An optimization problem consists of an objective function which may either represent the efforts and resources required or the desired results. If the function represents the efforts then it is required to be minimum and if results then it needs to be maximum .Response Surface Methodology is a useful technique to perform optimization having a varied domain of application including biological processes and should be used effectively in the studies to obtain conditions for most promising results. The above study strongly supported the use of Response Surface Methodology in different biological processes including media Optimization for fermentation studies.

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