Isolation, Identification, Characterization of *Bacillus Subtilis Subsp Subtilis.*, Producing the Keratinase Enzyme Under Optimization Method

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ABSTRACT *The aim of this study to isolate the keratinase producing bacteria from poultry farm, slaughter house. In that, feather dumping soil (100 samples) was collected from several area includes the Coimbatore, Pollachi, and Erode. Among these samples, the eighteen isolate were subjected for primary screening using Milk casein agar plate. From these isolates, the Bacillus subtilis subsp subtilis., showing a highest keratinase activity in secondary screening using Casein agar medium & its confirmed by 16s rRNA gene sequence. The specific organism subjected to keratinase activity assay and it's identified by the spectrometric using the keratin azure as a substrate. Thus, this organism was optimized by Response enzyme methodology (RSM) using the production medium. In this, fructose shows a 100% result for the keratinase activity;enzyme activity about 8+/- 1.9; pH 6.5, temperature 370C with incubation of 7 days under OGM and dextrose shows an important role in both biomass production and keratinase activity. The keratinase production was favored in the presence of ammonium tartarate as the nitrogen source. The optimum level of glucose for about 5.61g/l; enzyme activity was around 13.280 U /ml. In same way, the keratinase production was favored in the presence of ammonium tartarate as the nitrogen source; the enzyme activity in yeast extract (nitrogen source) as 9.8+/-1.3. Simultaneously, keratinase production were selected based on the initial screening experiments involving carbon and nitrogen source using glucose, starch, starch and ammonium tartarate, glucose and ammonium tartarate, OMG with ammonium tartarate under the submerged fermentation, among the glucose and ammonium tartarate range from 11.2+/-0.3 (keratinase activity)U/ml.*

1. Introduction:-

Keratin occurs in nature mainly in the form of hair, feather, wool, nail, horn. Feather as a waste product annually, which consists of approximately 90% keratin (Santos et al., 1996)

In the present study a simple medium was employed to elaborate large quantities of extracellular Keratinase from Bacillus subtilis subsp subtilis .The growth of cells and the accumulation of metabolic products are strongly influenced by medium compositions such as carbon sources, nitrogen sources, growth factors, and inorganic salts. Optimization of enzyme production can be carried out using The Response surface methodology (RSM). RSM is a collection of statistical techniques for designing experiments and searching optimum conditions of factors for desirable responses (Oh et al., 1995; Shieh et al., 1995; Sunitha et al., 1999) either in a full- or a fractional-factorial design that allows the testing of multiple independent process variables within a set of experiments. It has been successfully applied to the optimization of culture media for the production of primary and secondary metabolites in many fermentation processes (Li et al., 2002). Normally the conventional method used for such multifactor experimental procedure is by the 'change of one factor at a time' method. This method may lead to unreliable results and wrong conclusions, and it is inferior to the factorial design method (Plackett & Burman 1944; Srinivas et al., 1994). Thus RSM, which includes factorial designs and regression analysis, can better deal with multifactor influence on experiments towards optimizing the conditions for keratinase production

2. Materials and methods

Soil sample (Soil and feather) were taken from the natural composting in the poultry shop at Coimbatore City. Serial dilution from each sample was prepared by adding 1gm of the soil sample to the 9ml of distilled water. The diluted water plated on Nutrient Agar medium and incubated at 35C for 24hour. From the isolated colonies, the culture is inoculated by streaking method on Casein agar plates. The identified keratinase exhibiting bacteria are confirmed in 16s rRNA sequence and its an Bacillus subtilis subsp subtilis. Keratinase

activity determined by using keratin azure as substrate. The Keratin azure was suspended in Carbonate buffer (10mM, pH-10) at concentration of 4mg/ml. The reaction mixture contained 1ml of enzyme and 1ml of keratin azure suspension. The sample was inoculated at 37C, 300rpm for 1hour. After incubation, the mixture was kept in ice for 15 minutes followed by centrifugation at 5000rpm for 15 minutes to remove unutilized substrate. The supernatant was spectrophotometrically measured for the release of the azodye at 595nm.Keratinase activity for Bacillus subtilis subsp subtilis is 980 U/ml. A control was kept with enzyme and buffer without substrate.

3. Experimental design and optimization

The range of variables investigated is given in table 2. A 23 fractional factorial central composite design for three independent variables each at five levels, with ten star points and six replicates at the centre points leading 20 experiments was used in this study (table 3). In developing the regression equation the independent variables were coded according to the equation $Xi = (Zi-Zi^*)/\Delta Zi$. Where Zi stands for the uncoded value of ith independent variable, Zi^* denotes uncoded value of ith independent variable at center point and ΔZi is a step change value.

The DESIGN–EXPERT package [Design Expert (Software) ver 2.05, Stat-Ease, Inc. Hennepin square, Suite 191, 2021 East Hennepin avenue, Minneapolis, MN 554113] was used for regression and graphical analysis of the data obtained.

4. Results and Discussion:

The effects on growth and enzyme production of the replacement of dextrose in optimal growth medium (OGM) with various carbon sources were studied .It was noted that highest keratinase production by Bacillus subtilis subsp subtilis occurred in OGM .

(1) The carbon sources in the medium play an important role in enzyme production for about 10+/- 1.2 keratinase activity (U/ml). However, in the present study, dextrose played an important role in influencing both keratinase

and biomass production simultaneously. Culture conditions: The initial medium pH (6.0) and incubation temperature was 37°C for 7 days. The composition of Optimal growth medium is potato starch, 5 g l-1 and glucose, 5 g l-1 .

(2) The effects on growth and enzyme production of Bacillus subtilis subsp subtilis with glucose as fixed carbon source and varying the nitrogen source (Urea,KNO2, NaNO3 etc) were studied. Along with , the nitrogen source ammonium tartarate plays a major role and shows an Keratinase activity (U/ml) for about 10.2+/-0.2 sin submerged fermentation.

(3) Based on the medium components, this experiments was carried out glucose, starch, OMG and ammonium tartarte were selected as sole carbon and nitrogen source for optimization of keratinase production using RSM. The enzyme production was found to be maximum on 6th day after inoculation. In these medium, the Glucose and ammonium tartarate shows about 11.2+/-0.3 keratinase activity.

(4) The keratinase production (6th day; henceforth called yield, Yj) was taken as the dependant variable. Experimental range and levels of independent nutrient variables

Z Variables (g l-1)	Coded variables	Variable Levels					Step change value ΔZi
		-2	-1	0	+1	+2	
Glucose	X1	1.5	3.0	5.5	8.0	10.5	2.5
Ammonium tartarate	X2	2.0	4.0	6.0	8.0	10.0	2.0
Sodium acetate	X3	2.0	4.0	6.0	8.0	10.0	2.0

(5). Response surface statistical technique resulted in the second order polynomial equation, which relates the keratinase activity at the 6th day of growth to the tested variables in coded unit. Response surface statistical technique resulted in the second order polynomial equation, which relates the keratinase activity at the 6th day of growth to the tested variables in coded unit.(T1 to T20 experpiments) here some of the negative and positive value are tabulated.

Experiment	Coded variables			Keratinase activity (U ml-1)		
	X1	X2	X3	Actual data (Yj)	Predicted data (Ŷj)	Residual value
T1	-1	-1	-1	8.403	8.961852	-0.558852
T3	-1	+1	-1	9.262	10.171852	-0.909852
T5	-1	-1	+1	10.61	10.918852	-0.307852
T9	-2	0	0	9.871	9.199.23	0.671977
T12	0	+2	0	10.985	10.060273	0.924727

(6) It can be seen that the variables with higher effect were squared terms of glucose concentration ($X1^2$) and sodium acetate ($X3^2$) followed by interaction terms X1, X2 and X1*X3. Higher significance of the squared terms ($X1^2$, $X2^2$ and $X3^2$) over corresponding linear terms (X1, X2 and X3) shows that the optimum values for keratinase production lies within the experimental values chosen

Significance of regression coefficients

Variable	Regression coefficient	Computed t value	Significance level, p value
Constant	13.209591	43.63	
X1	0.081125	0.4275	0.6781
X2	-0.160500	-0.8458	0.4175
X3	0.324250	1.709	0.1183
X1*X2	-0.732750	-2.730	0.0212
X1*X3	-0.621500	-2.316	0.0431
X2*X3	-0.032750	-0.1220	0.9053

5 .Conclusion

At these optimum values, the maximum predicted keratinase activity was 13.280 U ml-1. These optimum values were checked experimentally which resulted in keratinase activity

which corresponded to 12.80 U ml-1 of enzyme activity. The experimental results correspond to the 96.5 % of the predicted value. The good correlation between these two results verifies the validity of the response model and the existence of an optimal point. The Response surface methodology was employed in optimizing the nutrient levels needed towards the optimal production of keratinase enzyme by A Bacillus subtilis subsp subtilis 23 factorial central composite experimental design was used. The multiple regression equation, relating the enzyme activity to the nutrient medium, was used to find the optimum values of glucose, ammonium tartarate and sodium acetate. The optimum values of these variables for maximal enzyme production were found to be: glucose, 5.61 g l-1; ammonium tartarate, 5.72 g l-1 and sodium acetate, 6.32 g l-1 with the predicted enzyme activity of 13.280 U ml-1.

Diagrammatic presentation for glucose and ammonium tartarate, glucose and sodium acetate. phosphate and ammonium tartarate
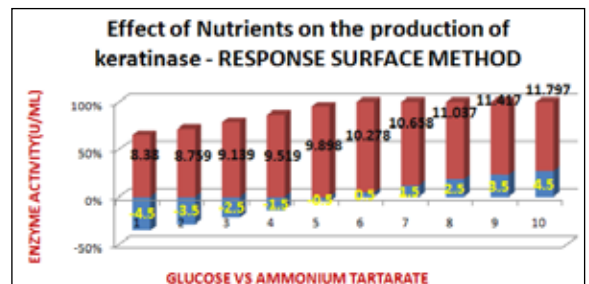

Figure1. Effect of nutrients on the production of Keratinase response surface plot (upper) and its contour plot (lower) of interaction between glucose and ammonium tartarate.
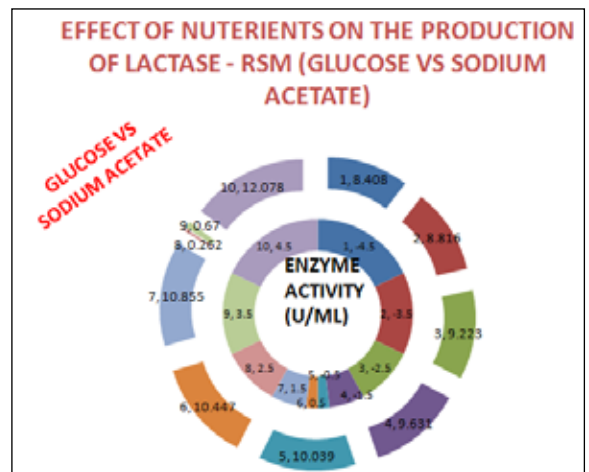

Figure 2. Effect of nutrients on the production of lactase: response surface plot (upper) and its contour plot (lower) of interaction between glucose and sodium acetate.
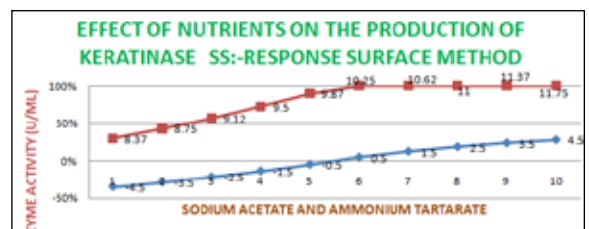

Figure 3. Effect of nutrients on the production of Keratinase ss: response surface plot (upper) and its contour plot (lower) of interaction between phosphate and ammonium tartarate

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