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Statistical analysis on optimisation of Microbial Keratinase enzyme screened from Tirumala and Tirupati soil samples

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Abstract

Due to the availability of powerful expression systems in various microbes, the large scale commercial production of new and useful enzymes is becoming increasingly attractive (10.11), The development of microbial enzymes for commercial use is a specialized field which requires.Continuous screening of new and improved strains from potential sources.Scaling up of enzyme production by optimizing conditions of fermentation(1,2,3,4,12,14) .Strain improvement of the isolated strains by classics or by recombinant methods of genetic manipulation. Statistical analysis, Down stream purification technology and formulation of enzymes(12,13,15). The biochemical heterogenicity and ecological diversity of microbes including actinomycetes and their exceptional capacity of producing secondary metabolites make them an obvious target as the source of new enzymes. They are considered potential sources of proteases with typical substrate specificities such as keratinases which attack the normally unreactive keratin(7,8). Microbial keratinases play a key role in leather processing, (5),bioconversion of keratinous waste materials to feed supplement, or source of amino acids. Thus keratinase producing organisms have both economic and ecological value (6,7,8,14). Studies on statistical analysis of keratinase have not been fully exploited hence it is addressed as the primary objective of the present study.

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Keratinase, Tirumala, Keratin, Statistical analysis

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INTRODUCTION

Tirumala is being visited by thousands of pilgrims every day from all parts of India as well as whole world, all round the year to worship Lord Venkateswara. (5.6.7) The place has relatively high deposits of keratin as compared to any other place as most of the pilgrims coming to Tirumala consider head tonsuring as the most sacred act of offering to

God. Further the prevailing climatic conditions also facilitate the growth of thermo-tolerant organisms.(1,2,3,4,9) Thus this place was presumed to provide good enrichment for potential keratinophilic and keratinolytic organisms and soil samples collected from various locations in Tirumala Hills and Tirupati were analyzed to isolate keratinolytic organisms. Statistical analysis was carried out through SPSS 10.1 package

MATERIALS AND METHODS

Studies of the comparison of keratinase production in three different media showed that the keratinase production was highest in production media(8,9,10,11). Hence production media was selected as the medium of choice for optimization of nutritional as well as physical parameters, including type and concentration of carbon source, nitrogen source, effect of pH, temperature, agitation on keratinase production using the six test strains.

***Statistical analysis*:** Statistical analysis was carried out through SPSS 10.1 package and p levels at 0.05 were considered as significant. Initially descriptive statistics was carried for the target variable keratinase enzyme production with respect to the effect of the type of carbon and nitrogen source, physical parameters of growth like pH, temperature, agitation, duration of the fermentation and the strains (17),. The enzyme production was not showing variation with respect to duration of fermentation in different strains as the keratinase production reached its maximum yield by 240-288 hrs. of fermentation in most of the cases. Hence, further analysis was confined to other variables only. Chi-square (χ^2) values strengthened the use of univariate analysis and logistic regression to know the interactions as well as relative importance of one variable over the other. For the analysis of variance highest keratinase enzyme activity was taken as dependent variable and nutrient source and type of strain as factors. For physical parameters like pH, temperature and agitation also, the keratinase yield was taken as dependent variable and pH and strain; temperature and strain; agitation and still conditions in relation to strains, were taken as factors (9,12,13,&17). The analysis was carried out independently for carbon source, substitution of nitrogen source, addition of nitrogen source, pH, temperature and agitation.

For the logistic regression, a multinominal full factorial model was fitted with type of sugar as dependant variable and keratinase enzyme activity as covariate. Subsequent controls of the type of strain in the model, did not alter the odds ratio and hence only keratinase enzyme activity was retained in the model. The factorial model was obtained for parameters like carbon source, substitution of nitrogen source, addition of nitrogen source, pH, temperature and agitation etc. A low yielding keratinase enzyme condition as specified in each case, was taken as reference in the analysis. From the logistic analysis, those variables with significant yielding ($P<0.05$) when compared to the reference were selected and applied for Tukeys harmonic mean comparison for identifying the theoretical combination that yielded maximum keratinase activity(16,17). The best combination was also studied for fermentation to check the relevance of theoretical model taking into account the cost factor of the nutrients also.

RESULTS AND DISCUSSION

Effect of the carbon source on production of Keratinase: Galactose, the carbon source in production media was replaced with equivalent concentration of different carbon sources, to study the influence of carbon source on keratinase production. The results and corresponding statistical analysis are summarized in Tables -1-8. The results indicate that glucose supported highest keratinase production with the test strains followed by galactose and starch. Lactose and glycerol were next in order.

The ANOVA and other statistical analysis revealed that the type of sugar as well as the type of strain and their interactions were significant. Glucose gained a odds ratio of 2.022, followed by 1.616 and 1.392 by galactose and starch respectively (Table-1a & 1b).

Table 1a: Anova of the Data on Effect of Carbon Source on Keratinase Production.

Sugar	Type III Sum of squares	Df	Mean square	F	Sig.
Type of sugar (I)	2287.804	6	381.301	49.128	P< .001
Type of strain (II)	148.452	5	29.690	3.825	P< .001
I X II	703.648	30	23.455	3.022	P< .001
Error	1629.871	210	7.761		
Total	18793.680	252			

Table 1b: Multinominal Logistic Regression Model for Comparison of the Type of Carbon Source on Keratinase Production

Variable	Odds ratio	95%C-I		Sig.
Glucose	2.022	1.652	2.475	P<.001
Galactose	1.616	1.350	1.934	P<.001
Starch	1.392	1.172	1.654	P<.001
Mannitol	0.878	0.720	1.072	P = NS
Lactose	1.049	0.877	1.256	P = NS
Maltose	1.154	0.970	1.376	P = NS

Note: Glycerol was taken as reference

$\chi^2 = 147.408$ p< .001

NS = Not Significant.

Table 2a: Anova of the Data on Effect of Substitution of Nitrogen Source on Keratinase Production

Nitrogen source	Type III Sum of squares	Df	Mean square	F	Sig
Type of Nitrogen source (I)	270.838	4	506.849	81.245	P< .001
Type of strain (II)	314.810	5	78.475	12.579	P< .001
I X II	27.633	20	5.531	0.887	P< .001
Error	2881.108	150	6.239		
Total	27926.870	180			

Table 2b: Multinominal Logistic Regression Model for Substitution of Nitrogen Source on Keratinase Production.

Type of Nitrogen source	Odds ratio	95%C-I	Sig
Peptone	0.989	0.888 1.101	P=NS
Yeast extract	1.047	0.941 1.166	P=NS
Soyabean meal	1.187	1.058 1.331	P<0.01
Ground nut cake	1.109	0.994 1.237	P<0.07

Note: $(NH_4)_2HPO_4$ is taken as reference for fd

$\chi^2 = 147.479$ p< .001

NS : Not significant

Designing of fermentation media for optimum production of keratinase: The carbon and nitrogen sources which were showing significance in the logistic regression model were grouped by descriptive statistics for keratinase activity (Table-3). Further, the Tukey's harmonic mean for sample size with alpha level significant at 0.05, was carried out and categorization is shown in Table- 4. The yielding of keratinase in the subset one was nearly identical for starch and galactose among carbon sources and $(NH_4)_2 HPO_4$ as nitrogen source. Similarly in the subset 2, groundnut cake, and soyabean meal among nitrogen sources and glucose among carbon source are almost identical. The results of comparison of the designed fermentation media based on the statistical analysis on keratinase production taking KLH_{104} and KLF_{16} strains showed that the media with glucose as carbon source and soyabean meal as nitrogen source was only marginally better than media with starch as carbon source and soyabean meal as nitrogen source

Table 3: Descriptive statistics for the Keratinase enzyme activity for the highest yielding based on the logistic model

Source	N	Mean	S.D.	95% CI	
Carbon					
Glucose	36	13.43	4.15	12.03	14.84
Galactose	36	9.91	3.64	8.67	11.14
Starch	36	7.98	3.62	6.76	9.21
Nitrogen					
Soyabeen	36	13.60	4.94	11.93	15.27
Groundnut cake	36	12.43	4.40	10.95	13.92
$(NH_4)_2HPO_4$	36	10.52	3.38	9.38	11.67

Table 4: Means for groups in homogenous subsets for the Keratinase enzyme activity

Combination	N	Subset for alpha=0.5	
Set 1:			
Carbon source:			
Starch	36	7.98	
Galactose	36	9.91	
Nitrogen source: $(NH_4)_2SO_4$	36	10.52	
Set 2:			
Carbon source : Glucose			
Nitrogen source:	36		13.43
Groundnut Cake	36		12.43
Soyabean meal	36		13.60

Table 5: Anova of the Data on Effect of pH on Keratinase Production

pH	Type III Sum of squares	Df	Mean square	F	Sig
Range of pH (I)	4561.312	9	506.849	81.245	P< .001
Type of strain (II)	392.375	5	78.475	12.579	P< .001
I X II	248.878	46	5.531	0.887	P< .001
Error	1871.550	300	6.239		
Total	27483.760	360			

Table 6: Multinominal Logistic Regression Model for Comparison of effect of pH on Keratinase Production

Variable	Odds ratio	95%C-I		Sig
10	3.924	2.753	5.592	P<.001
9.5	3.973	2.786	5.667	P<.001
9.0	3.847	2.702	5.476	P<.001
8.5	3.532	2.492	5.005	P<.001
8.0	2.723	1.943	3.817	P<.001
7.5	2.025	1.453	2.822	P<.001
7.0	1.612	1.151	2.257	P=NS
6.5	1.359	0.962	1.920	P=NS
5.5	1.225	0.861	1.744	P=NS

Note: pH 4.5 is taken as reference
$\chi^2 = 340.189$ p< .001 NS = Not Significant.

Table 7: Anova of the Data on Effect of Temperature on Keratinase Production

Temperature	Type III Sum of squares	Df	Mean square	F	Sig
Range of temperature(I)	685.932	3	228.644	24.016	P< .001
Type of strain (II)	1628.329	5	325.666	34.207	P< .001
I X II	1365.652	15	91.043	9.563	P< .001
Error	1142.440	120	9.520		
Total	20119.920	144			

Table 8: Multinominal Logistic Regression Model for Comparison of Agitation on Keratinase Production

Variable	Odds ratio	95%C-I		Sig
Agitation	1.239	1.101	1.394	P<.001

Note: Still condition is taken as reference
$\chi^2 = 15.406$ p< .001

DISCUSSION:

The production and activity of enzymes are influenced by various physical and nutritional factors. In the present study, the optimum nutritional and physical conditions for production of keratinases were determined for the six isolates, identified to be producing more than 10 KU/ml in the initial study in production media

The univariate ANOVA of the data on the effect of carbon source on keratinase production revealed that the types of sugar as well as the type of strain and their interactions were significant statistically. The maximum keratinase enzyme activity in each of the cases found significant variance with the type of sugar as given by the corresponding F values (Table - 1a & 1b). In order to find out the relative yield of keratinase with respect to sugars, a multinominal logistic model was carried out taking type of sugar as dependent variable and enzyme activity as covariate. Glycerol the carbon source in basal media was taken as reference to derive the odds ratio. The results indicated that the production of keratinase was 2.022

folds higher followed by galactose and starch which were 1.616 and 1.392 folds more respectively when compared with glycerol, and were significant. Mannitol, lactose and maltose were insignificant on enzyme yield as compared to reference. When the type of strain was included in the model as covariate and the odds ratio value was not altered significantly and hence the results are not shown. No significant difference in the keratinase yield with the interaction of strains and fermentation duration was observed as most of the strains reached a maximum by 240- 288 hrs of incubation, after which there was a decrease in the production of enzyme.Multinominal logistic models and descriptive statistics explains the clearly the studies regarding keratinase enzyme production and paved the further improvement in the microbial keratinase studies.

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