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Isolation of Streptomyces Species from Soil and its Medium Optimization for Microbial Transglutaminase Production by Box-Behnken Design

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Abstract

Transglutaminase (E.C. 2.3.2.13) are a family of enzymes that catalyze the covalent bond formation between open amine groups. They are widely used in food industries and their demand rises daily. Though they are available in mammalian tissues, fish and plants, the complex separation and purification process led to the search of Microbial Transglutaminase (MTGase). Finding a new microbial source of transglutaminase and the medium composition for MTGase production were the purpose of this work. Six Different types of Actinomycetes like strains were isolated from soil sample and two of them named PG03 and PG06 were selected based on their ability to produce 23 mg/ml and 21 mg/ml MTGase enzyme respectively. Strain PG03 was chosen for further studies and it was found to be a *Streptomyces species*. Standard enzyme production media composition is modified and tested to facilitate the optimized MTGase activity. Strategies like finest nitrogen and carbon source selection, revealing the key ingredients of media by full factorial design and their optimal concentration Box-Behnken design were adopted. At the 95% confidence level, second order polynomial model was applied to fit the research outcome. Under the proposed optimized conditions, the model predicted a transglutaminase yield of 21.7 mg/ml, very closely matching the experimental value of 24.1 mg/ml. The F-test was greater than the table value of 2.82 and the p-value of 0.004 clearly reveals that this regression was statistically significant at the 95% confidence level. Further, the proposed model has the ability to elucidate 48.8% response variation as indicated by the R^2 of the regression value.

Keywords: Box-Behnken design; Medium optimization; Microbial transglutaminase; Plackett-Burman design; *Streptomyces sp.*

Introduction

Transglutaminase (TGase) is an enzyme that catalyses an acyl transfer reaction using peptide-bond glutamine residues as acyl donors and several primary amines as acceptors. When the ε-amino groups of the protein-bond lysyl residues are present as acyl receptors, this enzyme transglutaminase is capable of forming intra and intermolecular ε-(γ-Glu)-Lys isopeptide bonds [1]. The covalent cross-links between a number of proteins and peptides introduced by transglutaminase promote the modification of the food proteins [2]. Therefore transglutaminase catalyzed reactions may be broadly used by food processing industries, for instance, the creation of new product textures, the modification of viscosity, the alteration of emulsifying and foaming properties, and the product nutritional value [3,4].

Insoluble and extensively cross-linked protein polymers essential for the organism to create barriers like blood clots and stable structures like hair and skin are generally produced by transglutaminases. The catalytic reaction is intimately observed with extensive control system as it is irreversible [5]. The reaction catalyzed by Transglutaminase is given as follows:

Peptide-Lysine + Glutamine-Peptide $\xrightarrow{\text{TGase}}$ Peptide-Lysine=Glutamine-Peptide

Industrial Applications

Transglutaminase catalyzed reactions are broadly used by food processing industries in hot dogs, sausages, rationalized steaks for fastening little meat portions into a large one and in improving the quality of low-grade meat like pale, soft, and exudative (PSE) meat. It is also used in making milk and yogurt creamier and noodles stiffer. Transglutaminase is also used to produce some unusual foods.

Transglutaminases are found in mammalian tissues, plasma, fish and plants [6]. Eight transglutaminases have been characterized till date and they are Factor XIII (fibrin stabilizing factor), keratinocyte transglutaminase, Tissue transglutaminase, epidermal transglutaminase, Prostate transglutaminase, TGM X, TGM Y and TGM Z [5]. The mammalian enzymes are Ca^{2+}-dependent. However, the relatively small quantities obtained and the complex separation and purification procedures required for these enzymes led to the search for alternative microbiological sources. The first microbial transglutaminase (MTGase) characterized was from an Actinomycetes. Since then, efforts have been made to obtain massive production of this enzyme for commercial applications, especially for the enzymes from *Streptomyces.*

Streptomyces consists of a vegetative hyphae approximately 0.5 – 2.0 µm in diameter, producing an extensively branched mycelium that rarely fragments. Colonies at the outset are pretty smooth surfaced and soon after build up aerial mycelium weft that looks like powdery,

floccose, velvety or granular. They are gram positive but not acid-alcohol fast, catalase positive, degrade L-tyrosine, starch, hypoxanthine, gelatin, esculin, casein, adenine and nitrate reduction to nitrite. They are abundantly distributed and in soil as well as composts.

So far, research has been focused on the isolation and screening of microorganisms for transglutaminase activity, and on purifying and characterizing newly found enzymes. The media compositions used to produce microbial transglutaminase from *Streptomyces* have been almost the same in every work published since Ando et al. [7]. The formulation of the culture media is of grave importance in industrial biotechnology processes as it influences volumetric productivity, product yield and concentration. Reducing medium cost is vital as it impinge on the whole process economics.

The traditional one-at-a-time optimization strategy is simple and constructive in screening and the individual medium component effects are observed on a chart not including complicated statistical analysis.

As the combined effects on the response are not considered, this simple technique often fell short in finding the optimum response section. The lack of understanding about complicated interactions between different factors leads to complications and qualms related with large scale fermentation. Statistically based experimental designs provide an efficient approach to optimization. The Plackett-Burman Design is especially appropriate in determining the most important components in the medium that make up the interactions.

An amalgamation of factors producing an assured optimal response was identified through factorial design along with response surface methodology (RSM). It is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, significant interactions between variables can be identified and quantified by this technique.

Taking into account that the soil is a great reservoir of Actinomycetes and that there are few reports concerning optimization of culture medium for transglutaminase production, in this project, the isolation and screening of soil Actinomycetes for transglutaminase production is reported and the nutritional fermentation conditions in order to maximize the transglutaminase yield was studied.

Materials and Methods

Selective isolation and preservation of actinomycetes

The actinomycetes strains used in this study were isolated from soil samples collected from Trichy, Tamil Nadu. Nearly five grams of the soil sample were added to 10 ml sterilized distilled water and the suspension was shaken at 200 rpm for 10 min. Aliquots were inoculated onto Petri dishes containing Rose Bengal Agar and incubated for three days. Actinomycetes-like colonies were streaked onto slants of ISP2 media [8] and checked for purity. Colonies were removed from the agar media and preserved in deep freezer with 10% glycerol solution at -20°C.

MTGase-production screening

The ability to produce transglutaminase was determined by inoculating 1 ml of spore suspension into 250 ml Erlenmeyer flasks containing 50 ml of seed medium. The flasks were incubated for 2 days at 30°C and 200 rpm in a rotational shaker. Aliquots of 15 ml of pre inoculum were transferred to 135 ml of the basal medium [7] in 500 ml Erlenmeyer flasks and cultivated at 30°C for 5 days at 200 rpm. All runs were made in duplicate. After that, the amount of microbial transglutaminase activity was estimated.

MTGase Estimation

Aliquots of 1 ml of culture medium were taken and after centrifugation the enzyme amount was detected in the supernatant. Equal volumes (50 µl) of supernatant and transglutaminase reaction mix containing transglutaminase assay buffer with both donor and acceptor substrate and 1 M DTT, is taken in a 96 well plate, mixed and incubated for 2 hrs. at 37°C. After incubation, 50 µl of stop solution was added and mixed by pipetting and centrifuged at rpm for 15 min to pellet the precipitate formed. 100 µl of supernatant was transferred to a new 96 well plate containing hydroxamate standards and the colour developed was read at 525 nm. From the hydroxamate standard curve, the absorbance value is converted into nmole of hydroxamate which is then converted to mg/ml of hydroxamate using the molecular weight of hydroxamate. This value is used to represent the amount of transglutaminase produced in the entire article.

Identification of Species

The selected strain was identified by Gram's staining and confirmed by culturing in Starch-Casein Agar plates.

Screening of optimal carbon and nitrogen sources

The effects of different sources of nitrogen and carbon on the TGase production were scrutinized with classical one at time approach. The nitrogen sources and corresponding concentrations tested were: 2% peptone with 0.2% yeast extract; 2% peptone; 2% corn steep liquor (CSL); and 2% casein. The carbon sources were: 2% potato starch with 0.2% glucose; 2% molasses; 2% sucrose with 0.2% glucose; 2% glycerol; and 2% soluble starch. In the investigation of the nitrogen sources, growth was carried out in the medium containing: 0.2% KH_2PO_4; 0.1% $MgSO_4.7H_2O$; 2% soybean flour; 2% potato starch; 0.2% glucose. In the process of screening carbon sources, fermentation was carried out using the medium containing: 0.2% KH_2PO_4; 0.1% $MgSO_4.7H_2O$; 2% soybean flour and 2% peptone.

Elucidation of significant components by Plackett-Burman Method

10 ml of medium was prepared with the following components as shown in the Table 1 in distilled water. The mediums are then autoclaved and after cooling inoculated with 1 ml of strain. Incubation was done in a shaker at 37°C at 200 rpm for 72 hrs. After incubation the enzyme produced were estimated by transglutaminase assay method provided earlier and are analyzed using F-test [9].

Optimization of key ingredient concentration using RSM

The concentrations of key ingredients identified as a result of Plackett-Burman method were optimized using Response Surface Methodology (RSM), especially by Box-Behnken Design. The experiment was designed by NCSS 2007 software. The experimental design including the variables and concentration levels were shown in the following Table 1. The experimental results were analyzed using the StatistXL software and NCSS 2007.

Trial	Variable (%)							Yield (mg/ml)
	KH_2PO_4	$MgSO_4.7H_2O$	Glucose	Peptone	Yeast Extract	Sucrose	Molasses	
1	0.3	0.15	3.0	1.0	0.2	1.0	1.0	25.8
2	0.1	0.15	3.0	3.0	0.2	3.0	1.0	40.3
3	0.1	0.05	3.0	3.0	0.2	1.0	3.0	37.4
4	0.3	0.05	1.0	3.0	0.2	3.0	1.0	46.9
5	0.1	0.15	1.0	1.0	0.2	3.0	3.0	52.8
6	0.3	0.05	3.0	1.0	0.2	3.0	3.0	55.3
7	0.3	0.15	1.0	3.0	0.2	1.0	3.0	82.4
8	0.1	0.05	1.0	1.0	0.2	1.0	1.0	33.3

Table 1: Plackett-Burman design.

Result

The strategy involved in this study, comprising selective isolation conditions for actinomycetes from soil sample from Trichy, was successful for the recovery of around 6 actinomycete pure cultures. These isolates were investigated for transglutaminase production and the results (Figure 1) showed that more enzyme was produced by the strains PG03 (23 mg/ml) and PG06 (21 mg/ml). The former strain was chosen to continue the studies and was taxonomically identified as Starch and casein hydrolyzing, Gram positive *Streptomyces sp.*

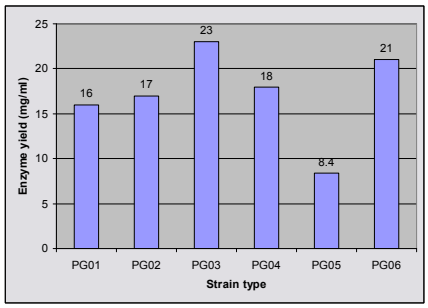

Figure 1: Transglutaminase production by various *Actinomycetes* strains.

The results of the effects of the carbon and nitrogen sources on the microbial transglutaminase production by *Streptomyces sp.* PG03 are shown in the Figure 2. Of all nitrogen sources investigated, the most promising was peptone and a mixture of peptone with yeast extract where the microbial transglutaminase production was around 27.4 mg/ml and 27 mg/ml respectively. Hence both peptone and yeast extract were taken for further studies. For the carbon source the best results were obtained with a mixture of glucose with sucrose (17 mg/ml) and molasses (18.3 mg/ml).

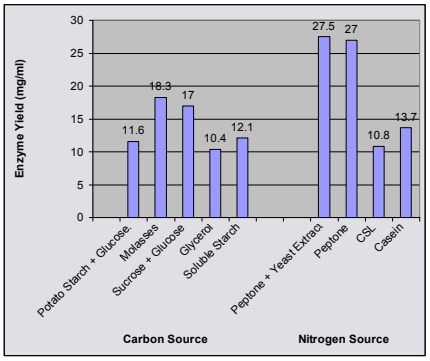

Figure 2: Selection of Carbon and Nitrogen Sources.

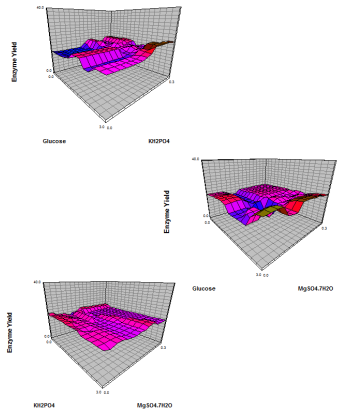

Figure 3: Response surface plots for the MTGase production as a function of: (a) Glucose versus KH_2PO_4; (b) Glucose versus $MgSO_4.7H_2O$; (c) KH_2PO_4 versus $MgSO_4.7H_2O$.

Since the outcome for both carbon supplies were not drastically diverse, glucose, sucrose and molasses were chosen to continue the

study. The most important nutrient factors were screened by Plackett-Burman Design as described earlier in Table 1. The experimental design and the results of Plackett-Burman design observations are presented in Table 1. Transglutaminase production varied from 25.8 mg/ml to 82.4 mg/ml with different combinations of the components in the media. The F-test for the design was given in the Table 2.

Response Surface Methodology, especially Box-Behnken design was chosen to optimize the key ingredients selected in the media. The experimental design and results are shown in the Table 3. The quadratic model calculated using NCSS for maximum microbial transglutaminase yield after eliminating the statistically insignificant terms was:

$$Y = 26.94444 - 9.8625x_1 - 222.75x_3 + 3.341667x_4 + 2.882292x_1^2 + 1137.917x_3^2.$$

Variable	KH_2PO_4	$MgSO_4.7H_2O$	Glucose	Peptone	Yeast Extract	Sucrose	Molasses
∑(H)	208.4	201.3	158.8	205.0	160.9	193.3	190.5
∑(L)	163.8	170.9	213.4	167.2	211.3	178.9	181.7
∑(H) - ∑(L)	44.61	30.4	-54.6	37.8	-50.4	14.4	8.8
Effect	11.15	7.6	-13.65	9.45	-12.6	3.6	2.2
Mean Square	248.75	115.52	372.64	178.60	317.52	25.92	9.68
F-test	0.78	0.36	1.17	0.5625	1.0	0.081	0.03

Table 2: F-test for Plackett-Burman design.

Trial	Glucose (%)	KH_2PO_4 (%)	$MgSO_4.7H_2O$ (%)	Peptone (%)	Yield (mg/ml)	
					Predicted	Experimental
1	1.0	0.1	0.10	2.0	15.7	13.3
2	3.0	0.1	0.10	2.0	19.1	17.1
3	1.0	0.3	0.10	2.0	15.7	19.1
4	3.0	0.3	0.10	2.0	19.1	21.6
5	2.0	0.2	0.05	1.0	13.8	11.2
6	2.0	0.2	0.15	1.0	14.3	15.8
7	2.0	0.2	0.05	3.0	20.5	20.0
8	2.0	0.2	0.15	3.0	21.0	19.5
9	2.0	0.2	0.10	2.0	14.5	16.2
10	1.0	0.2	0.10	1.0	12.4	11.6
11	3.0	0.2	0.10	1.0	15.7	15.8
12	1.0	0.2	0.10	3.0	19.1	17.9
13	3.0	0.2	0.10	3.0	22.4	19.1
14	2.0	0.1	0.05	2.0	17.1	15.4
15	2.0	0.3	0.05	2.0	17.1	17.9
16	2.0	0.1	0.15	2.0	17.6	20.0
17	2.0	0.3	0.15	2.0	17.6	15.4
18	2.0	0.2	0.10	2.0	14.5	16.2
19	1.0	0.2	0.05	2.0	18.3	20.0

20	3.0	0.2	0.05	2.0	21.7	24.1
21	1.0	0.2	0.15	2.0	18.8	18.3
22	3.0	0.2	0.15	2.0	22.2	22.5
23	2.0	0.1	0.10	1.0	11.2	08.7
24	2.0	0.3	0.10	1.0	11.2	10.8
25	2.0	0.1	0.10	3.0	17.9	20.4
26	2.0	0.3	0.10	3.0	17.9	17.1
27	2.0	0.2	0.10	2.0	14.5	16.2

Table 3: Response surface methodology.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	Ftest	p-value
Regression	172.118	4	43.030	5.253	0.004
Residual	180.222	22	8.192		
Total	352.341	26			

Table 4: Analysis of variance for Box-Behnken design.

The ANOVA obtained by StatistXL (Table 4) proved that the model was momentous. The F-test was greater than the table value of 2.82 [10] and the p-value of 0.004 clearly reveals that this regression was statistically significant at the 95% confidence level [11]. Further, the R^2 of the regression obtained was 0.488 indicating that 48.8% of the response variation can be elucidated by the model.

The response surfaces were selected with the probable mishmash to visualize the simultaneous effects of Glucose, KH_2PO_4, $MgSO_4.7H_2O$ and peptone on microbial transglutaminase production pattern.

Discussion

A *Streptomyces sp.* strain from soil sample was secluded as a potential producer of transglutaminase enzyme. It was as an extracellular calcium independent enzyme producer making it much more appealing and sensible for commercial applications [2].

Different compounds were examined with classical one at a time strategy to pick the best carbon and nitrogen sources for transglutaminase production. When peptone (27 mg/ml) and a mixture of peptone with yeast extract (27.4 mg/ml) were used as nitrogen sources and molasses (18.3 mg/ml) and a mixture of glucose and sucrose (17 mg/ml) as carbon sources, superior results were achieved. The results for both the carbon and nitrogen sources were not significantly different and hence all of them (Peptone, yeast extract, molasses, sucrose and glucose) were selected the Plackett-Burman design.

Variations in the concentration of molasses and sucrose in Plackett-Burman Design did not affect microbial transglutaminase production significantly. Considering this and the cost parameter, the concentration of sucrose is maintained at low level and molasses was not at all added. Conversely, KH_2PO_4, $MgSO_4.7H_2O$, Glucose and peptone affected the enzyme production to a greater extent. These four components were further investigated using Box-Behnken Design in a broader concentration range.

The results from the Box-Behnken Design showed that the concentrations of the four key ingredients were: 3% Glucose, 0.2% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 2% peptone. The maximum yield of microbial transglutaminase predicted with the model was 21.7 mg/ml whereas the experimental value was 24.1 mg/ml proving that the model was ample to forecast the optimization of transglutaminase production by *Streptomyces sp* PG03.

Conclusion

The fermentation media for microbial transglutaminase production have been modified and optimized via statistical methods like Plackett-Burman Design and Box-Behnken Design. The medium optimization not only resulted in an increased microbial transglutaminase production, but also reduced the constituent costs and an improvement in repeatability. Besides, the factors responsible for better activity were found and are imperative for further studies.

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Conflict of Interest Disclosure

The authors declare that they have no conflict of interest.

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