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RESEARCH PAPER

Screening and Statistical Optimisation of Media Ingredients for Production of Microbial Transglutaminase

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ABSTRACT

Transglutaminase is a calcium dependent enzyme that catalyses acyl transfer reactions between primary amino groups and protein bound glutamine residues. Eighteen bacteria and twenty eight actinomycetes were screened for the presence of transglutaminase. Among the microbial cultures screened *Streptomyces* sp. D1, showed maximum transglutaminase activity. In this study characterisation of transglutaminase. In this study characterisation of transglutaminase and its usefulness to cross link milk proteins was studied, using enzyme treated milk for preparation of paneer (Indian cottage cheese). Optimum temperature and pH for enzyme was found to be at 50 °C and 6.0, respectively. Optimisation of media ingredients for maximising the transglutaminase activity using *Streptomyces* sp. D1 was carried out by central composite design. Response surface methodology was employed to standardise the optimum media composition for maximum enzyme activity. Three factors such as carbon source, nitrogen source and pH were tested for the maximum enzyme activity as response. The optimised medium with sugarcane molasses as carbon source 6.0 per cent (w/v), peptone as nitrogen source 1.75 per cent (w/v) were found to be optimal at initial pH 6.5 and incubation temperature 30.0 °C with agitation at 100 rpm for 72 h. The enzyme activity of transglutaminase obtained from the optimised medium was found to be 4.1 (AU/ml). Low cost substrate such as sugarcane molasses in the form of a renewable substrate is proposed to be suitable for scale-up production of enzyme and for industrial applications. The ethanol fractionated enzyme treated milk produced increased amount of paneer with more water content and decreased cooking loss in comparison to the untreated control.

Keywords: Transglutaminase; Selective isolation; Screening; Identification; Food application

1. INTRODUCTION

Transglutaminase (*R*-glutaminyl-peptide: amineg-glutamyltransferase, EC 2.3.2.13) is a calcium-dependent enzyme that catalyzes the acyl transfer reaction between γ-carboxyamide groups of glutamine residues and ε-amino groups of lysine residues causing covalent cross linking between proteins, peptides and primary amines. When the acyl-acceptor is the ε-amino groups of lysine residues in proteins, intra- and inter-molecular isopeptide bonds are formed. In the absence of primary amines, water acts as the acyl-acceptor, causing the formation of glutamic acid residues by deamination of γ-carboxyamide groups of glutamine residues[1]. Transglutaminases (TGase) are widely distributed in nature and have been isolated from microorganisms, animal and plant tissues. The only commercial source of this enzyme available is obtained from *Streptomyces mobaraensis*[2] and microbial transglutaminases (MTGase) has gained importance due to simpler separation and purification processes in comparison to mammalian sources such as liver and other body fluids of vertebrates[3]. Recent applications of enzyme technology include covalent modification of protein structure without its cleavage. Possible applications of TGase in food industry has been explored due to its ability to form both inter- and intra-molecular cross-linking bonds in the proteins, restructing them and improving the physical properties of protein rich foods[4]. The possible food application of MTGase produced by *Streptomyces mobaraensis* established[5]. Applications of TGase for improvement of texture, water holding capacity, elasticity, nutritional value, and appearance of meat, fish, wheat and soybean products has been reported[6-12]. Modifications of physicochemical properties of dairy products and gluten for food applications and biomedical applications using TGase was reviewed[13-15]. MTGase have been reported in actinomycetes and bacteria such as *Streptomyces mobaraensis*[5,18], *S. ladakanum*[16], *S. hygroscopicus*[17], *Bacillus subtilis*[19] and *B. circulans*[20]. Modification of properties of novel proteins for food applications demands catalysts such as TGase but the high cost involved in production limits their application. About 30 per cent of the production cost represents the fermentation media used for microbial production so cheap agro industrial wastes such as sorghum straw, sugarcane molasses etc have been explored earlier as substrate for economical production of transglutaminase. The aim of the present study is to isolate transglutaminase producing microorganism, biotechnological production of enzyme using statistical optimisation in a medium containing sugarcane molasses a low cost agro industrial substrate. Furthermore, the produced enzyme was characterised

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and its application in cross linking of milk proteins was studied by changes in physical properties of paneer (Indian cottage cheese).

2. MATERIALS AND METHODS

2.1 Chemical and Reagents

All chemicals were of analytical grade. N-Carbo-benzoxy-L-glycine (CBZ), L-glutamic acid, hydroxylamine γ-monohydroxamate and glutathione were purchased from Sigma-Aldrich (Mumbai, India). Carbon sources such as fructose, galactose, lactose, maltose, xylose, and nitrogen sources such as casein peptone and protease peptone were procured from Hi-media (Mumbai, India). Sucrose, sodium chloride, yeast extract, and acetone were procured from SD Fine chemicals (Mumbai, India). Pasteurised milk (Nandhini Dairy, Mysore) was procured from local market and the composition of milk as given by the manufacturer is carbohydrates 4.8 per cent (lactose 4.0 per cent), fat 3.6 per cent, protein 3.3 per cent, calcium 124 mg, minerals 0.2 per cent and vitamin A 40 μg.

2.2 Microorganisms and Culture Conditions

Bacterial and actinomycete strains were isolated from soil and marine samples. The isolation was carried out by serial dilution of the samples followed by inoculation onto solidified starch casein agar medium of composition, starch 1 per cent, casein 0.1 per cent, agar 1.5 per cent; and pH 7.2 ± 0.2 and incubation at 30 °C for up to 5 days. Actinomycete like colonies with powdery and leathery appearance were streaked onto slants of yeast extract, malt extract agar media (ISP 2 sporulation media) of the composition, 0.4 per cent yeast extract, 1 per cent malt extract, 0.4 per cent of glucose and agar at pH 7.0±0.2 and the spore suspensions were used for further studies. *Streptomyces* and *Bacillus* strains procured from IMTECH, Chandigarh including *Streptomyces albereticuli* MTCC 323, *S. olivoverticilla* MTCC 333, *S. griseocarneus* MTCC 328, *S. septatus* MTCC 926, *Bacillus* sp. MTCC 864 and MTCC 1434 were also screened for enzyme production. Freeze-dried culture of wild *Strptomyces*sp. NRRL-287 strain was provided by the Agricultural Research Service Culture Collection (Peoria, Illinois, USA). The cultures were revived in sterile water and grown in ISP Medium 2 for spore formation before inoculating into production media. The microorganisms used in this study also included *Bacillus* species such as *B. mycoides* DF1 and *B. Subtilis* DFR40 from Food Biotechnology Division, DFRL, Mysuru, India.

2.3 Screening of Microorganisms for Transglutaminase Production

Actinomycetes and bacterial strains were screened for presence of transglutaminase on the Petri plate using filter paper disc (FPD) assay[21]. In this method 30 mm FPD were placed on single actinomycetes colony grown on medium comprising of glucose 0.5 per cent, poly peptone 2.0 per cent, soluble starch 2.0 per cent, K_2HPO_4 0.2 per cent, K_2HPO_4 0.2 per cent, $MgSO_4$ 0.01 per cent, yeast extract 0.2 per cent and agar 1.5 per cent at pH 7.0 and on bacterial colonies grown on medium comprising of poly peptone 2.0 per cent, soluble starch 2.0 per cent, K_2HPO_4 0.2 per cent, $MgSO_4$ 0.01 per cent, yeast extract 0.2 per cent and agar 1.5 per cent at pH 7.0. 30 μl of substrate comprising of 125mM hydroxylamine, 12.5 mM glutathione (reduced - GSH-) and 37.5 mM N-carbo-benzoxy-L-glycine in 200 mM citrate buffer (pH 6.0) was added to FPD placed over the colony and incubated at 37 °C for 1 h. The reaction was stopped by adding 10 μl of 5 per cent $FeCl_3$ in 15 per cent Trichloroacetic acid. The change in colour of FPD from white to burgandy brown indicates the presence of transglutaminase. The actinomycete strain positive for enzyme activity was identified by morphology and microscopy using Gram staining procedure using Himedia Grams Stain-Kit. The strain was also characterised biochemically for the utilisation of carbohydrates such as lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, arabinose and mannose using Himedia HiCarbo Kit.

2.4 Inoculum Preparation and Production of Transglutaminase

The actinomycete culture inoculum, showing positive reaction in FPD assay for transglutaminase, was prepared by transferring a loopful of the inoculums into 50 ml seed medium in a 100 ml Erlenmeyer flask containing (g/L) glucose 4.0, yeast extract 4.0, malt extract 10.0 with an initial pH of 7.2 to 7.4. The inoculated medium was kept for 48 h at 30±2 °C under static conditions. Production of the polymer was carried out in 250 ml capacity Erlenmeyer flasks containing 100 ml medium. The production medium comprising (g/L) soluble starch 0.2, glucose 0.05, peptone 0.2, yeast extract 0.12, $MgSO_4 7H_2O$ 0.01, KH_2PO_4 0.02, and K_2HPO_4 0.02 was inoculated with 2 per cent (v/v) of 48 h old actinomycetes seed culture. The inoculated flasks were kept at 30°C on a rotary shaker at 100 rpm 35±2 °C for 72 h. After incubation for 72 h the cell biomass was separated by centrifugation at 10000 rpm for 15 min and the enzyme from supernatant was precipitated overnight using 80 per cent acetone at 4 °C. The protein fraction was collected by centrifugation at 10000 rpm for 15 min at 4 °C.

2.5 Enzyme Assay

The protein fraction obtained by ethanol precipitation was assayed for protein concentration and transglutaminase activity. Reaction mixture containing 1ml of substrate containing 125 mM hydroxylamine, 12.5 mM glutathione (reduced - GSH-) and 37.5 mM N-carbo-benzoxy-L-glycine in 200mM citrate buffer (pH 6.0) was added to 0.5 ml of acetone precipitated enzyme and incubated at 37 °C for 1h. The reaction was stopped by adding 0.5 ml of 5 per cent $FeCl_3$ in 15 per cent TCA and e absorbance was measured at 525 nm using UV-vis Spectrophotometer (UV- 1601, Shimadzu). One unit of transglutaminase activity was defined as the amount of enzyme that causes the formation of one micromole of hydroxamic acid per min at 37 °C. L-glutamic acid.γ-monohydroxamate was used for preparing the calibration curve.

2.6 Effect of Temperature and pH on Transglutaminase Activity

Optimum temperature for the activity of transglutaminase was determined by incubating the reaction mixture at 10.0-

90.0 °C in 200 mM citrate buffer, pH 6.0, using 125mM hydroxylamine, 12.5 mM glutathione (reduced - GSH-) and 37.5 mM N-carbo-benzoxy-L-glycine as the substrate. Temperature stability of the enzyme was tested by pre-incubation for 30 min at different temperatures in the range 30-100 °C at pH 6.0 and the relative activity of transglutaminase was measured. The pH stability of transglutaminasewas assessed by pre-incubating the enzyme for 12 h at 4.0 °C with buffered sucrose ranging from pH 4.0 to pH 12.0, pH 4.0 and pH 5.0 (Sodium acetate buffer), pH 6.0 pH 7.0, pH 8.0 (Phosphate buffer) and pH 9.0, 10.0, 11.0 and 12.0 (Glycine –NaOH buffer) at 4 °C. The residual enzyme activity was determined under standard assay conditions. The transglutaminase activity of the pre incubated sample at 4 °C was taken as 100 per cent.

2.7 Selection of Carbon and Nitrogen Source for Transglutaminase Production

The influence of different carbon sources such as molasses, sucrose, glycerol and soluble starch were studied at 2.5 per cent (w/v) by replacing carbon source in the basal medium. The effect of nitrogen sources on transglutaminase production were also studied using peptone, yeast extract, corn steep liquor and casein at 2.5 per cent. The pH of the media was adjusted to 7.0 by the addition of 1 N NaOH prior to autoclaving. 45 h culture was inoculated at 2 per cent (v/v) and fermentation was carried out at 30 °C for 72 h under agitation at 100 rpm in 250 ml Erlenmeyer flasks containing 100 ml media.

2.8 Central Composite Rotatable Design

To maximize the transglutaminase production from the isolated actinomycetes strain a combination of suitable physical parameters were identified based on 'one-at-a-time' approach followed by central composite rotatable design (CCRD) using RSM (Minitab software, Version, 14). CCRD is an experimental design which enables to fit a first or second-order polynomial by a least significance technique. This design facilitates in locating the optimum point within the unknown region of interest and also ensures the uniformity of the magnitude of prediction error for all points at the same radial distance from the centre point [22].The CCRD for three independent variables such as sugarcane molasses (X_1, per cent), peptone (X_2, per cent) and pH (X_3) each at three levels leading to a total number of 40 experiments was employed for optimisation. Each variable was studied at two different levels (-1, +1) and center point (0) which is the midpoint of each factor range. In developing the regression equation, the test factors were coded according to the following equation:

$$x = X_i - X_0/\Delta X_i \quad (1)$$

where x is the dimensionless coded value, X_i is the actual value of variables, X_0 is the actual value of variables at the centre point, and ΔX is the step change value. The experimental results were fitted with a second-order polynomial function:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (2)$$

where Y is the predicted response, b_0 the model constant, b_1, b_2, b_3 the linear coefficient, b_{11}, b_{22}, b_{33} the squared coefficient and b_{12}, b_{13}, b_{23} the interaction coefficient.

2.9 Data Analysis

Minitab software, Version 14[23] was used for the data analysis. The response surface model graphs were used to identify the effects of linear, quadratic and interactive terms of the independent variables on the chosen dependent variables. To validate the model, the average of each response (enzyme production) was determined from the completely optimised medium composition in duplicates. The statistical significance of the model was checked by Fischer's F test and the level of significance was given as p value.

2.10 Application of Transglutaminase in Preparation of Paneer

250 ml of homogenised cow's milk containing 3.5 per cent (w/v) total fat was boiled at 100 °C for 10 min and allowed cool at room temperature. 5 ml of enzyme (4.2 AU/ml) was added slowly to the milk with constant stirring and refrigerated at 4 °C for 18 h. Enzyme treated milk samples were boiled and 35 ml of 10 per cent (w/v) citric acid solution was added slowly with constant stirring till clear whey separated out. The curd was left to settle down for 10 min without any agitation. For preparation of paneer (Indian cottage cheese) the curdled milk was filtered using a muslin cloth and hung for 2 h to remove excess water. The paneer obtained was made into a square shaped block by compressing using approximately 2 kg weight over the paneer for 1 h. Before analyses the samples were placed on a wooden plank for 10 min to 15 min to ensure draining of excess whey from the prepared paneer. Yield of paneer (g) total water content (per cent) and cooking loss (per cent) were estimated. Total water content of paneer sample was estimated by measuring the initial weight of the paneer (W_1) before placing in oven at 100 ± 2 °C for 16 h. The samples were cooled to room temperature and the final weight was measured (W_2). The total water content of paneer was calculated using the formula and expressed in percentage.

Water content (per cent) = $(W_1 - W_2) / W_1 \times 100$

Cooking loss of paneer samples was estimated according to Nonaka[24], *et al*. The samples of size 3.0 cm x 1.5 cm x 1.5 cm were immersed in boiling water for 15 minutes with an initial weight (W_1). The paneer samples were filtered through a muslin cloth and cooled to room temperature, and final weight was measured (W_2). The cooking loss of paneer was calculated using the below formula and expressed in percentage.

Cooking loss (per cent) = $(W_1 - W_2) / W_1 \times 100$

3. RESULTS AND DISCUSSION

3.1 Screening and Identification of Microorganisms for Transglutaminase Production

Selective isolation of actinomycetes from soil samples resulted in recovery of 23 actinomycetes in pure culture form and selective isolation of bacteria from marine samples resulted in 17 pure cultures. These isolates were investigated for TGase production using FPD and hydroxamate assay. The culture which produced transglutaminase showed colour change of FPD from white to burgundy brown while cultures that did not produce transglutaminase remained white. The results indicated that highest enzyme activity was found in the actinomycete strains D1 (0.46 AU/

Table 1. Actinobacterial and bacterial cultures showing transglutaminase activity in FPD and hydroxamate assay

Microorganism	FPD assay	Hydroxamate assay (AU/ml)
Actinomycetes		
Isolate D1	Positive	0.463
Isolate H2	Positive	0.260
Isolate 2B2	Positive	0.235
Isolate 2G2	Positive	0.189
Streptomyces albereticuli MTCC 323	Positive	0.154
S. griseocarneus MTCC 328	Positive	0.240
Bacteria		
Isolate D40	Positive	0.069
Isolate M12	Positive	0.078
Isolate M16	Positive	0.098
Bacillus sp. MTCC 1434	Positive	0.830
Bacillus sp. MTCC 864	Positive	0.123

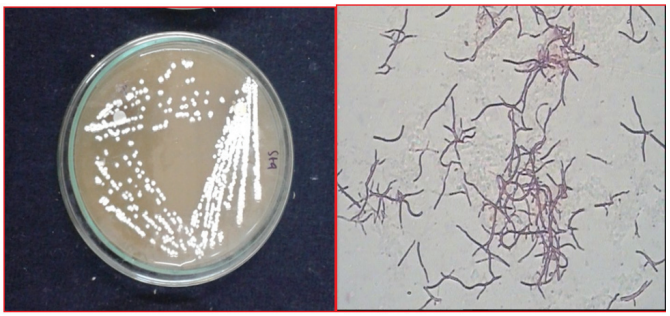
ml) and H2 (0.26 AU/ml) using the hydroxamate assay (Table 1). The strain D1 was chosen to continue the studies for production and optimisation of transglutaminase and identified morphologically by round, powdery white colonies and microscopically as gram positive *Streptomyces* sp. (Fig. 1). The positive strain for transglutaminase production *Streptomyces* sp. D1 utilised carbohydrate such as lactose, xylose, galactose, raffinose, trehalose, melibiose, sucrose, arabinose and mannose while could not utilise maltose, fructose and dextrose

3.2 Characterisation of Transglutaminase

The optimal conditions for enzyme activity in terms of temperature and pH for the enzyme activity were investigated based on the hydroxamic acid formed by the enzyme from substrate. The influence of temperature on transglutaminase activity was examined at pH 6.0, and the optimum temperature was found to be 50 °C. The enzyme activity at 40 °C was 60–70 per cent and less than 40 per cent above 60 °C. The temperature stability drastically reduced on incubation above 50 °C for 20 min (Fig. 2). Ando[5], *et al.* reported TGase from *S. mobarensis* with optimum temperature of 50-60°C. The relative activity of enzyme increased above pH 5.0 and retained 60 per cent of its activity over the pH range 5.0–10.0 even after 18-h incubation and the optimum pH was found to be 6.0 (Fig. 3 and 4). The activity decreased rapidly for alkaline pH. Similar optimum pH were reported for transglutaminase produced using *S. hygroscopicus* and *S. ladakanum*[16,17]. *S. mobarensis* DSM40585 TGase with an optimum pH of 6.0 and optimum temperature of 50 °C was also reported[25].

3.3 Effect of Carbon and Nitrogen Source on Transglutaminase Production

Sugars such as sugarcane molasses, glycerol, sucrose and soluble starch were used as carbon source for enzyme production. Among the carbon sources used sugarcane molasses supported highest enzyme production of 1.3 (AU/ml) while glycerol, sucrose and soluble starch produced enzyme below 0.2 (AU/ml) as estimated by hydroxamate assay. Sugarcane molasses a by-product of the sugar cane industry contains monosaccharides



(a) (b)

Figure 1. (a) Colony morphology and (b) Gram staining of *Streptomyces* sp.D1.

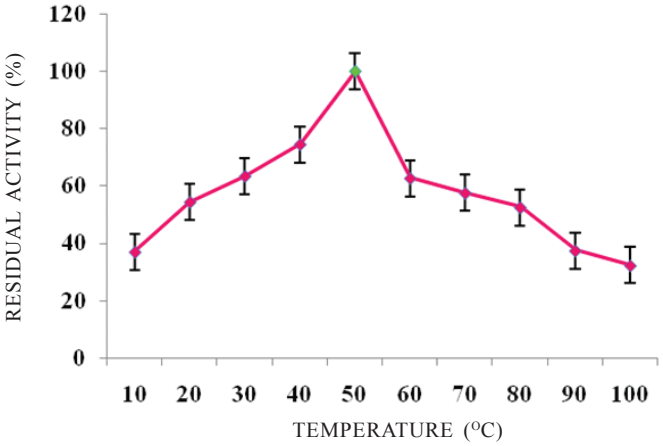


Figure 2. Effect of temperature on transglutaminase activity. 100 per cent relative activity is equal to 4.10 AU/ml.

(glucose and fructose) and a disaccharide (saccharose) in high concentrations. Sugarcane molasses resulted in the enzyme production at higher level which could be due to presence of potassium, chloride, sulfur, calcium, and sodium salts[26] or trace metals such as iron, zinc, and copper[27] supporting *Streptomyces* growth and enzyme production. Biotechnological applications for sugarcane molasses have been reported for the production of

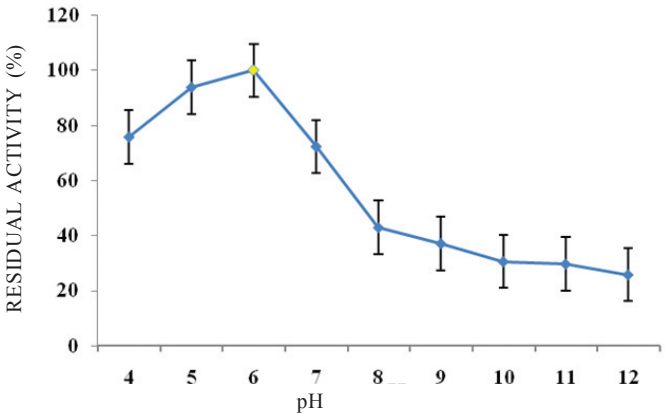


Figure 3. Effect of pH on the enzyme activity of transglutaminase. 100 per cent relative activity is equal to 4.10 AU/ml.

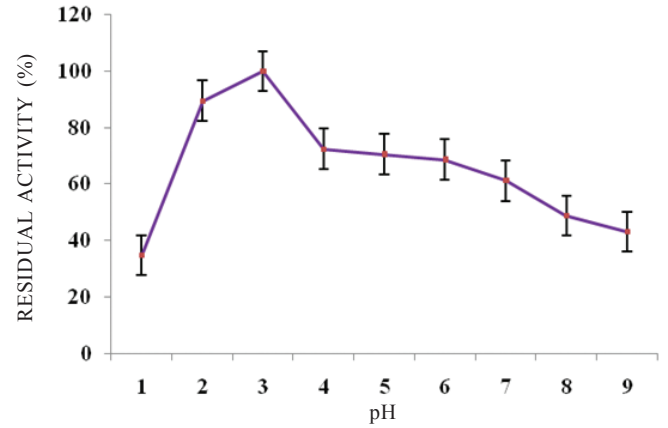


Figure 4. Effect of pH on the enzyme stability of transglutaminase. 100 per cent relative activity is equal to 4.10 AU/ml.

various additives and enzymes such as transglutaminase by *S. ladakanum* NRRL3191[28], β-D- galactosidase by *Kluyveromyce smarxianus*[29], glucosyltransferase by *Erwinia* sp[30], ethanol by *Zymomonas mobilis*[31], lactic acid by *Enterococcus faecalis*[32], citric acid by *Aspergillus niger*[33] and sorbitol by *Zymomonas mobilis*[34]. Among the organic nitrogen sources tested at 2 per cent (w/v), peptone supported the highest enzyme production (1.3 AU/ml) when used as the sole nitrogen source in comparison with protease peptone and yeast extract.

3.4 Optimisation of Selective Medium Components Using Central Composite Design

In the present study the relationship between the response functions and process variables was identified by three-factor inscribed composite design. Production of transglutaminase at 30 °C for 72 h under agitation at 100 rpm was determined from previous one-factor-at a time experiments. Further, variables such as sugarcane molasses as carbon source, peptone as nitrogen source and suitable pH of the media for maximizing the production of transglutaminase were optimised by central composite design using RSM. The predicted and experimentally measured responses for 40 runs according to the experimental design are shown in Table 2. Enzyme activity ranged from 0.4 to 4.1 (AU/ml) and the maximum activity was obtained for the 38th run under the experimental conditions of X_1 (carbon source)=6.0 per cent; X_2 (nitrogen source)=1.75 per cent and X_3 (pH)=6.5. The lowest enzyme activity was obtained for the 9th and 10th runs with the following conditions of X_1(carbon source) =3.5 per cent; X_2(nitrogen source) =1.75 per cent and X_3(pH)=6.5 and also for 15th and 17th runs with the following conditions of X_1(carbon source)=1.96 per cent; X_2(nitrogen source) =0.98 per cent and X_3(pH) =8.0. Based on these data, the media components were optimised for obtaining desirable response i.e. enzyme activity at maximum.

3.5 Fitting the Model

The second order polynomial model equation was obtained by fitting the results obtained as shown in Eqn 3.

$$\text{Transglutaminase activity (AU/ml) (Y)} = 1.64 + 0.496x_1 + 0.096x_2 + 0.1493x_3 - 0.162x_1^2 + - 0.077x_2^2 - 0.317x_3^2 + 0.125x_1x_2 - 0.013x_1x_3 - 0.125x2x_3 \quad (3)$$

Analysis of variance (ANOVA) results indicated that the model is significant (Table 3). The significance of each regression coefficient was determined from the *p*-value of *F*-test ($p<0.05$). A lower *p*-value indicates the more significant nature of the corresponding coefficient[35]. Overall, a close relationship between the experimental values and the predicted values indicated that the developed model is satisfactory. The coefficient of regression (R^2) determines the quality of fit of the model and the R^2 values for enzyme activity (AU/ml) was 0.919. The model was confirmed as significant at the level of 0.0001 per cent probability level with the R^2> 83.70 per cent and adjusted R^2 of 76.40 per cent, where the value of R^2 > 75 per cent indicates the aptness of the model. This value ensured a satisfactory adjustment of quadratic model to explain the experimental data and indicated that the model could explain 90 per cent of the variability in the response (Table 4). The plot of expected versus residual values of enzyme yield also proves the significance of the model as the entire points cluster around the diagonal lines (Fig. 7).

3.6 Effect of Media Components and pH on Levan Yield

Transglutaminase production with maximum enzyme activity by the isolated strain of *Streptomyces* sp. D1 was optimized using the medium containing sugarcane molasses as the carbon source and peptone as the nitrogen source by CCRD. The fermentation conditions of 48 h incubation at 30 °C with agitation at 100 rpm obtained in preliminary experiments were kept uniform during the optimisation process. In the present study, the factors influencing the maximum enzyme activity were determined by the significant co-efficient of the second-order polynomial regression equation. The results indicated that the first-order linear effect was significant for sugarcane molasses concentration (X_1) and pH (X_3); second-order quadratic effect was significant for pH (X_3); interactive effect was significant for sugarcane molasses concentration (X_1) and pH (X_3) (Table 4). The data indicated that an increase in sugarcane molasses concentrations led to enhanced transglutaminase activity. A linear increase in enzyme activity was noticed with increasing concentration of sugarcane molasses, and a maximum yield of 4.1 (AU/ml) was obtained in the higher region of sugarcane

Table 2. Experimental design matrix in terms of actual, coded factors and the observed values for the response (Enzyme production AU/ml)

Independent variables	Units	Symbol	Coded levels				
			- α(axial)	-1	0	+1	+ α(axial)
Sugarcane molasses	per cent	X_1	1.0	1.9	3.5	5.03	6.0
Peptone	per cent	X_2	0.5	0.98	1.75	2.5	3.0
pH		X_3	4.0	5.0	6.5	8.0	8.9

Standard order	Run order	Coded variables			Response (*Y*) Enzyme activity (AU/ml)		Residual error
		X_1 Sugarcane molasses (per cent)	X_2 Peptone (per cent)	X_3 pH	Actual value	Predicted value	
1	1	5.03092	0.98454	8	1.5	1.1	0.28
2	2	1.96908	2.51546	8	0.6	0.1	0.28
3	3	5.03092	2.51546	8	1.4	1.3	0.28
4	4	1.96908	0.98454	8	0.5	0.4	0.28
5	5	5.03092	2.51546	5	2.1	1.9	0.28
6	6	1.96908	0.98454	5	0.7	0.4	0.28
7	7	1.96908	2.51546	5	0.8	0.6	0.28
8	8	5.03092	0.98454	5	1	1.2	0.28
9	9	3.5	1.75	6.5	0.4	0.9	0.16
10	10	3.5	1.75	6.5	0.4	0.9	0.28
11	11	3.5	1.75	6.5	0.5	0.9	0.28
12	12	3.5	1.75	6.5	0.9	0.9	0.28
13	13	1.96908	2.51546	5	0.8	0.8	0.28
14	14	1.96908	0.98454	5	1.2	0.6	0.16
15	15	1.96908	0.98454	8	0.4	0.5	0.28
16	16	3.5	1.75	6.5	1.1	1.1	0.28
17	17	1.96908	2.51546	8	0.4	0.2	0.28
18	18	5.03092	2.51546	8	1	1.5	0.16
19	19	3.5	1.75	6.5	1.1	1.1	0.28
20	20	3.5	1.75	6.5	1.3	1.1	0.28
21	21	3.5	1.75	6.5	1.2	1.1	0.16
22	22	5.03092	2.51546	5	2.2	2.0	0.28
23	23	5.03092	0.98454	8	1.2	1.3	1.35
24	24	5.03092	0.98454	5	1.2	1.3	1.91
25	25	3.5	1.75	6.5	2.4	1.9	1.96
26	26	3.5	0.5	6.5	1.4	1.9	1.91
27	27	3.5	1.75	6.5	2.2	1.9	0.19
28	28	1	1.75	6.5	0.7	1.5	0.28
29	29	3.5	1.75	8.9495	1.3	0.8	0.28
30	30	3.5	1.75	4.0505	1.2	1.3	0.28
31	31	6	1.75	6.5	3.1	3.1	0.28
32	32	3.5	3	6.5	2.6	2.3	0.28
33	33	3.5	1.75	6.5	2.9	2.5	0.19
34	34	3.5	0.5	6.5	2.9	2.6	0.28
35	35	3.5	1.75	6.5	3.1	2.5	0.19
36	36	1	1.75	6.5	2.2	2.2	0.28
37	37	3.5	3	6.5	2.3	3.0	0.28
38	38	6	1.75	6.5	4.1	3.8	0.28
39	39	3.5	1.75	8.9495	0.9	1.5	0.28
40	40	3.5	1.75	4.0505	1.6	1.9	0.28

Table 3. Regression analysis (ANOVA) for enzyme production

Regression	DF	SS	MS	F-value	p-value (Prob>F)
Model	3	7.39	2.46	3.57	0.000
Residual	36	24.86	0.69		
Total	39	32.26			

SS = Sum of square; DF = Degree of freedom; MS = Mean square R^2 = 83.70 per cent

Table 4. Analysis of regression coefficients and their significance for the response-enzyme production (AU/ml) (Y1)

Term	Co-efficient	t-value	p-value
Constant	1.6405	12.826	0.000
Sugarcane molasses	0.4958	1.130	0.000
Peptone	0.0967	1.130	0.268
pH	-0.1492	-1.744	0.093
Sugarcane molasses x Sugarcane molasses	0.1615	1.878	0.071
Peptone x Peptone	0.0771	0.897	0.378
pH x pH	-0.3166	-3.6810	0.001
Sugarcane molasses x Peptone	0.0125	1.131	0.268
Sugarcane molasses x pH	0.0125	0.113	0.991
Peptone x pH	-0.1250	-1.131	0.268

molasses 6.0 per cent (v/v), and thereafter below this level it was found to decrease.

The pH of the medium was also found to be a critical factor on enzyme activity. An increase in sugarcane molasses concentration along with pH 6.5 resulted in the increase of enzyme activity. A maximum activity of 4.1 AU/ml was obtained with pH 6.5. The increase in pH more than 6.5 coupled with increase in sugarcane molasses concentration from 3.5 to 5.1 per cent (v/v) decreased enzyme activity. It was also observed that increasing the concentration of peptone in combination with sugarcane molasses does not show increase in enzyme activity. The effect of interactive factors of sugarcane molasses and pH on enzyme production is shown in the 3D response surface and the 2D contour plots (Fig. 5 and 6) as a function of sugarcane molasses and peptone. The 2D contour and 3D response surface plots are the graphical representation of the regression equation. The interaction between the variables can be inferred from the shapes of the surface plots. Enhanced production of transglutaminase (4.01 AU/ml) by *Streptoverticillium ladakanum* NRRL 3191 in a response surface methodology optimised medium containing sugarcane molasses 60.0 (g/L) and glycerol 60.0 (g/L) as the sole carbon source at pH 7.0 was reported earlier[28]. Since the nitrogen source was a non-significant variable the 3D plot is shown at its zero level of X_2.These data are indicative of significant interaction in the limit of 95 per cent ($p>0.05$), between the concentration of sugarcane juice and peptone. The highest activity obtained according to the CCRD was higher

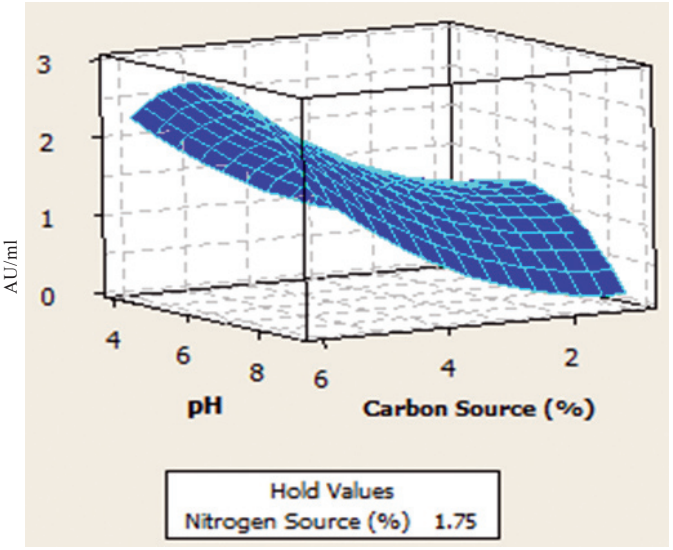


Figure 5. Effect of molasses and peptone on transglutaminase production by *Streptomyces* sp. D1.

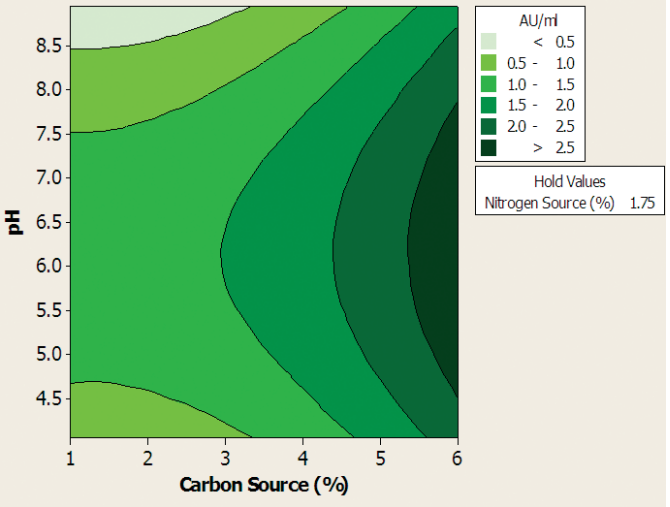


Figure 6. Contour plot depicting the effect of molasses and peptone on transglutaminase production by *Streptomyces* sp. D1.

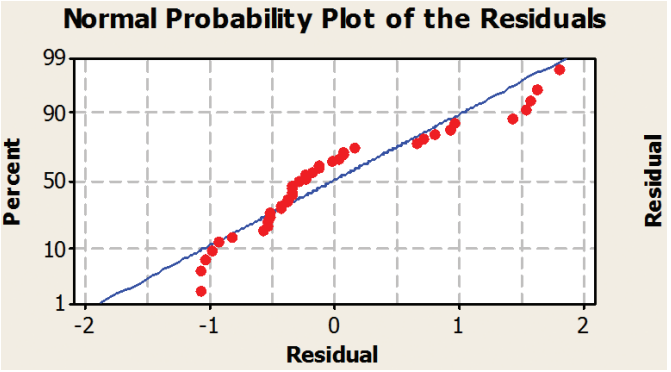


Figure 7. Plot of expected normal values versus residuals.

than the activity obtained during one at a time experiments. The excellent correlation between predicted and measured values confirms the response model and the existence of an optimal point[16,17].

3.7 Effect of Transglutaminase Activity on Paneer

The effects of the TGase produced using *Streptomyces* sp. D1 on yield, total water content and cooking loss of paneer (Indian cottage cheese) was studied. Effect of TGase treatment on paneer is shown in Fig. 8 100 ml of enzyme treated milk yielded paneer of 18.24 g on wet weight basis while the yield of paneer in control was 11.38 g on wet weight basis. The yield of paneer made from enzyme treated milk was 32.15 per cent higher when compared to the control (Fig. 8). Higher yield and enhanced water content of cheese made from *S. mobarensis* TGase treated milk was reported[36]. The cooking loss of paneer prepared using TGase treated milk was 4.3 per cent lesser than untreated milk. The increase in gel stability due to the enzymatic modification and enhanced even and homogenous distribution of milk proteins could be attributed to improved functional properties of dairy products[37]. The effect of adding MTGase (100 U g^{-1}) on the cottage cheese making process studied and 13 per cent increase in yield was observed using enzyme incubated for 90 min. The cottage cheese produced with MTGase showed textural changes such as firmness, stickiness, gumminess, and chewiness which were lower than those of the control product without added enzyme[38].

The total water content of paneer made from TGase treated milk was 9.5 per cent while that of untreated control was 7.4 per cent. The moisture content of enzyme treated paneer increased up 2.14 per cent when compared to untreated control (Fig. 8). Increased moisture content, yield and protein content using TGase treated milk in production of white brine cheese was reported earlier[39]. The decreased syneresis and more serum retention is due to the formation of a gel matrix with a lower pore size due to alterations in the physical characteristics of TGase treated casein gels[36]. Cooking loss of paneer during cooking may be due to the loss of water and other soluble compounds, due to the structural changes that take place during cooking which leads to release of the entrapped moisture. Cooking loss was 4.04 per cent for the paneer prepared from TGase treated milk while the untreated sample encountered a cooking loss of 5.76 per cent. Both the enzyme treated and control samples lost weight after cooking but the cooking loss for paneer made from enzyme treated milk was 29.8 per cent lesser than the untreated control (Fig. 8). The protein network formed while preparing paneer using TGase treated milk suggested that the protein network formed is more cohesive which is capable of holding more water during cooking, thereby reducing the cooking loss of paneer. Our findings are similar to earlier studies on tofu (paneer made from soy milk)[24,40].

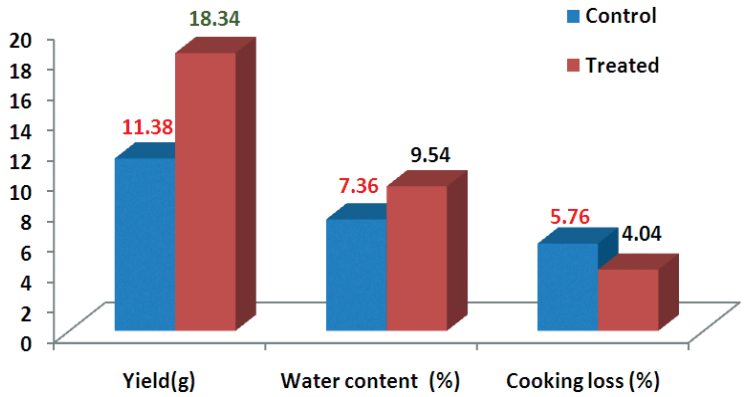


Figure 8. Comparison of yield, total water content and cooking loss of transglutaminase treated and untreated paneer.

4. CONCLUSION

Streptomyces sp. D1 isolated from soil samples was found to produce capable of transglutaminase that could be used as a catalyst for cross linking of proteins. The enzyme production was also optimised using low cost agro industrial substrate such as sugarcane molasses which resulted in higher enzyme yield. The study also indicates a valuable application of sugarcane molasses, as a low-cost substrate for further scale up of enzyme production and its application in food processing applications. Results presented in our study demonstrated the possible application of TGase obtained to improve the properties of paneer. The microbial transglutaminase in cost-effective bioprocess expands for its application as a cross linker agent for the production by production edible film as food wrapper and products using animal protein.

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