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Optimization of Induction Media for Production of Chondroitinase from *Proteus penneri* SN5 Using Response Surface Methodology

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Abstract

The objective of present study was to optimise the induction media for isolation of important therapeutic enzyme- chondroitinase from novel species *Proteus penneri* SN5 with the help of central composite design (CCD) of response surface methodology (RSM) through Design Expert 7.0 software. The reported source of origin of this bacterium is snake. Quadratic model was fitted to enhance the wet cell mass concentration of *P. penneri* SN5. The optimized induction media composition with RSM is (in g/l): nicotinic acid 0.10, yeast extract 0.95 and chondroitin sulphate 2.985 at pH 6.55. The wet cell mass yield was increased in optimized medium from 0.802±0.0067 g/100ml to 1.0847±0.0033 g/100ml. Chondroitinase was isolated by pulverization of cell mass with phosphate buffer containing tween 20, precipitated by ammonium sulphate and purified by cation exchangers. Protein concentration was found as 0.89±0.31 mg/ml and 0.57±0.17 mg/ml in crude and purified condition, respectively. Specific activity of chondroitinase was increased to 370.38±0.21 U/mg in purified form. The optimum pH was 8.0 for residual enzyme activity and found stable up to 40ºC temperature.

Keywords: *Proteus penneri*, Fibroproliferative Disorders, Response Surface Methodology, Chondroitinase, Chondroitin sulphate.

INTRODUCTION

Chondroitin sulphate proteoglycans (CSPGs) are the essential factors for routine functioning of the body while any changes in the level of normal CSPGs results in different fibro-proliferative diseases (FPDs). Glycosaminoglycans (GAGs) are linear acidic polysaccharides comprising a repeated units of a hexosamine linked to an uronic acid. GAGs are having the presence at both in the Extra-cellular matrix (ECM) and at surface of cells as proteoglycans' constituents. These sugars are participated in collagen tissue formation and maintenance throughout tissue homeostasis process and also fundamental modulators of many biological processes, such as development, cell proliferation, signaling and inflammation [1, 2, 3].

Proteus vulgaris is a representative species among *Proteus* genus which produces Chondroitinase (Chase ABC I and II) [4]. These enzymes enhance depolymerisation of variety of GAG substrates, including Chondroitin 4-sulphate (C4S), Chondroitin 6-sulphate (C6S), Dermatan sulphate (DS) and Hyaluronic acid (HA) which are major constituents of different scars, keloids, lesions and related skin proliferation [5]. Chase I and II enzymes are also having their extensive role in hydrolysis of different glycosaminoglycans in neural cells affected by spinal injury and intraspinal disc herniation [6]. Other chondroitinase producing genus *viz. Vibrio, Flavobacterium, Micrococcus, Aeromonas,* and *Arthrobacter* are with a varied range of Chondroitin sulphate (ChS) isomer degradation [7].

Various researches shown that *Proteus mirabilis* and *Corynebacterium acnes* produce Chase, but the extent of ChS isomer degradation was not determined [7,8]. Most nosocomial infections are originated by *P. mirabilis* (about 70-90%) and *P. vulgaris* and rarely by *P. penneri. P. penneri* is frequently being confused as *P. mirabilis* since both species confirm indole test negative on the grounds of morphological and biochemical tests [9].

Hickman *et al.,* [10], were the first who proposed the name *Proteus penneri sp. nov.* for a class of organisms formerly called *P. vulgaris* indole negative or *P. vulgaris* biogroup 1; which shows *P. penneri* possesses most similar characteristics to that of *P. vulgaris* including chondroitinase ABC production [11]. It is also hypothesized that *P. penneri* strain contains PROPEN_02299 (length: 1016 aa) gene involved in production of chondroitinase ABC glycosaminoglycan lyase [http://uniprot.org and http://skybase.c2b2.columbia.edu]. It is also noteworthy to mention that earlier attempt for production of chondroitinase ABC from *Proteus penneri* was successful with absolute enzyme activity rate [12]. Hence it was interested to determine chondroitinase enzyme production from *P. penneri* since the species showing over 70% identity concerned with domains, aliphatic index, instability index, isoelectric point (pI), and molecular weights of enzyme to *P. vulgaris* by phylogenetic analysis via BLASTP search.

MATERIALS AND METHODS

Bacterial strain and culture conditions

All chemicals used were analytical grade and procured from Sisco Research Laboratories Pvt. Ltd., Mumbai. Yeast extract and nutrient media were purchased from Hi-media, Mumbai while nicotinic acid and chondroitin sulphate-A were obtained as gift sample from Geltec Pvt. Ltd. Bangalore, India. *Proteus penneri* SN5 was obtained from Lokmangal Biotechnology College, Wadala, Maharashtra (India) and it was delivered in frozen glycerol solution. It was reported that the bacterial strain was originally isolated from snake while recieving. Bacterial cells were first propagated on Nutrient agar, incubated at 37°C for 24 h. [13]. The arisen colonies were harvested by glycerol-salt buffer solution and put in a series of 2 ml Cryogen tubes. These tubes were frozen immediately at -20°C for 24 h followed by further storage as *P. penneri* SN5 cell bank at -80°C for further use [14]. For each experiment, 1 cryogenic vial from *P. penneri* SN5 cell bank (containing 1 ml) was used to inoculate the previously sterilized 100 ml of nutrient broth (1.0% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose) with pH 7.2 in 250 ml Erlenmeyer flask which was further incubated at 30°C for 14 h. The culture was prepared with combinations of the independent variables. All experiments were performed in 250 ml Erlenmeyer flasks composed of 100 ml of the growth medium. After inoculation, the flasks were kept on the rotary shaker at 200 rpm for 14 h for incubation. The wet cell mass further harvested and then washed once with chilled 0.85% saline solution to obtain cultured wet cells [15].

Induction medium for chondroitinase production

The cultured wet cells (of about 0.5 g) were re-suspended in 100ml of previously prepared and sterilized induction medium containing 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.1 mg/ml nicotinic acid, 0.1% yeast extract, 0.3% chondroitin sulphate, pH 6.0 to 7.0 and incubated at 30°C for 9 h with shaking [15].

Optimization of process parameters for induction medium

The cultured wet cells of *P. penneri* SN5 were grown on sterile induction medium. Purity of cultured cells was confirmed by negative citrate test and ability of fermentation of sucrose and maltose sugars. The impact of various parameters in the medium including concentration and interaction among each other was studied, by varying one factor at a time. At each step, the variable was selected from the previous experiment to enhance induction medium conditions for optimum yield of *Proteus* wet cell mass and subsequently the chondroitinase enzyme [16].

Experimental design

On the basis of independent experiments, process variables were identified and further used to enhance wet cell mass of *P. penneri* SN5 by optimisation of induction media. RSM is a most exploratory software tool to substantiate the interaction and influence among variables on biochemical activities [17]. Central composite design (CCD) using Design Expert Software trial version 7.0, statistical software (State-Ease Inc., Minneaopolis, MN, USA) was applied to optimize induction medium for high yield wet cell mass of *P. penneri* SN5. A quadratic model was derived by a multiple regression approach for four independent variables *viz.* nicotinic acid, chondroitin sulphate, yeast extract and pH. Further it was evaluated at five different levels to determine the interactions between and among these variables at different levels to opt highest yield of *Proteus* cell mass and ultimately optimised chondroitinase enzyme production as shown (Table 1).

Table 1. Variables and their levels for wet cell mass production by *P. penneri* SN5.

Independent variables	Variable name	Levels				
		Axial (-α)	Lower (-1)	Central (0)	Higher (+1)	Axial (+α)
A	Nicotinic acid (mg 100 ml^{-1})	8	9	10	11	12
B	Yeast Extract (mg 100 ml^{-1})	90	95	100	105	110
C	Chondroitin sulphate (mg 100 ml^{-1})	200	250	300	350	400
D	pH	5.5	6.0	6.5	7.0	7.5

During CCD experiments, concentrations of K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$ and an incubation temperature of 30ºC for 9 h with aerobic conditions were kept as constant factors. Thirty experiments were exercised in CCD to determine curvature and effects of interaction among selected variables and significance concerned with the applied model was further examined by *F*-test with the goodness of fit by multiple correlations R and verification of R^2 coefficients.

Experimental validation of statistical model

Based on obtained results in statistical RSM analysis, the optimized induction medium was induced with cultured cell mass of *P. penneri* SN5 earlier grown on nutrient media and further incubated at 30ºC for 9 h. The wet cell mass was collected to estimate its weight and further processed for isolation of chondroitinase enzyme and its quantification studies [18].

Statistical analysis

One way ANOVA analysis was performed and the obtained results were presented as mean ± standard deviation of three triplicate experiments. The F-value at p <0.05 was used as the standard to determine statistical significance.

Harvesting and purification of chondroitinase enzyme

The cells received after induction (about 10 g from 1 litre induction media) were harvested by pulverization using 100 ml of 5 mM phosphate buffer solution (pH 7.0) with the help of a high-shear homogeniser (Remi Labs, India) for three min at 12,000 rpm by maintaining in ice-bath to make a suspension. 3% of tween 20 solution was mixed to enhance the extraction efficiency and further centrifuged in cooling centrifuge (Remi Labs, India) at 9000 rpm for 10min at 4ºC to obtain suspension comprised with chondroitinase enzyme. A 5% solution of protamine sulphate was mixed to a final concentration of 0.5% to eliminate nucleic acid and other proteins to the said suspension. The suspension was agitated at 4° C for 30 min and the precipitate was separated by centrifugation to obtain a supernatant. The crude chondroitinase was precipitated with 60% ammonium sulphate solution at cold condition and further isolated by centrifugation at 9000 rpm for 10 min. Enzyme was dialysed by constituting in water using 10K MWCO dialysis membrane (Thermo Fisher Scientific Inc., USA) and purified on ion exchange columns initially on CM-Sepharose (Merck, USA) and later on SP-Sepharose (Merck, USA) column by charging and equilibration with 5 mM phosphate buffer solution (pH 7.0). Later the column was eluted with the same buffer solution containing 0.1M NaCl to obtain enzyme fractions which were later pooled and lyophilised [15].

Determination of protein content

The protein content of the unknown sample was quantified by Lowry method using Bovine Serum Albumin (BSA) as a protein standard [19].Various dilutions of BSA solutions were prepared by adding stock BSA solution (1 mg/ ml) and water in the test tube. The final volume in each of the tubes was 5 ml and BSA concentration range from 0 to 200 µg /ml. From these different dilutions, a 0.2 ml protein solution was pipette out to another test tubes and 2 ml of alkaline copper sulphate reagent was added. The solution was mixed well and incubated at room temperature for 10 min. Further 0.2 ml of reagent Folin-Ciocalteau solution was mixed to each tube and again incubated for 30 min. The spectrophotometer was adjusted with blank and the absorbance of samples was determined at 660 nm on a Systronics-104 visible spectrophotometer.

Absorbance versus protein concentration curve was plotted to obtain a standard calibration plot. Finally concentration of unknown protein sample was determined by using the standard curve plotted [20].

Determination of chondroitinase activity

Chondroitinase activity (Chase lyase) was measured on the basis of increased absorbance at 232 nm. This method is a modified method as described by Yamagata *et al.* group [21]. To determine enzyme activity reagent A was prepared by using 0.05% (w/v) BSA in 250 mM Tris HCl and 300 mM sodium acetate Buffer with pH 8.0 at 37°C. To prepare reagent B, 50 ml of 50 mM potassium chloride solution was prepared in deionised water and pH adjusted to 1.8 at 25°C with 1 M HCl, 5 ml of 0.5% (w/v) chondroitin sulphate A solution in reagent A was used as substrate and 0.01% (w/v) BSA was prepared in deionised water. Chase enzyme solution was diluted in cold 0.01% BSA solution. Experiment was performed in three replicate where 0.8 ml of diluted enzyme solution was taken in test tube which further equilibrated to 37°C for 2 min, 0.2 ml of substrate solution was added followed by mixing thoroughly. It was incubated at 37°C for 20 min, later the reaction was stopped by heating the solutions for 1 min in a boiling water bath. 0.1 ml of incubated mixture from each tube was transferred to separate tubes containing 0.9 ml of Reagent B (KCl). The blank is carried out without enzyme. Each tube was incubated for an additional 10 min at 37°C, centrifuged for 10 min and transferred the Test and Blank supernatants to suitable quartz cuvettes. The absorbance was recorded at 232 nm for each tube on a Systronics-2202 PC based Double Beam UV VIS spectrophotometer.

Enzyme activity in U /ml was calculated by equation **(A)**,

$$\mathrm{U/ml} = \frac{\mathrm{Abs} \times 1 \times \mathrm{DF}}{\mathrm{EmM} \times 0.10 \times 0.8} \qquad \textbf{(A)}$$

Abs= Absorbance at 232 nm
DF = Dilution factor
EmM = Millimolar extinction coefficient of substrate chondroitin sulphate A: 5.1
0.10 = Volume (in ml) of reaction mix used
1.0 = Total volume (in ml) of assay
0.8 = Volume (in ml) of enzyme used
Specific Enzyme activity in U /mg was calculated by equation **(B)**,

$$\mathrm{U/mg} = \frac{\mathrm{U/ml\ of\ enzyme}}{\mathrm{mg/ml\ of\ protein\ content}} \qquad \textbf{(B)}$$

Unit definition: One unit will liberate 1.0 µmole of 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-ene-pyranosyluronic acid)-4-O-sulpho-D-galactose from chondroitin sulphate A or 1.0 µmole of 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-ene-pyranosyluronic acid)-6-O-sulpho-D-galactose from chondroitin sulphate C per minute at pH 8.0 at 37 ºC. [Quality control test procedure, http://www.sigmaaldrich.com].

Effect of temperature on chondroitinase activity

The purified enzyme was dissolved in 0.01% (w/v) BSA Solution which was later preincubated in a Tris HCl buffer solution (pH 7.0) for one hour at the desired temperature (10°C, 20°C, 30°C, 40°C and 50°C). After incubation, the enzyme activity was assayed as per method described earlier [15].

Effect of pH on chondroitinase activity

To determine the effect of pH on the enzyme activity, Chondroitinase enzyme solution was diluted in cold 0.01% BSA solution and redissolved in 0.5 ml Tris-acetic acid buffer (with desired pH value 5.0 and 6.0), in 0.5 ml of Tris HCl buffer (with desired pH value 7.0 and 8.0) and in 0.5 ml of glycine buffer (with desired pH value 9.0 and 10.0). The enzyme solutions were stabilized at 25° C for 24 h. It was then incubated at 37°C for 1 h before mixing with the substrate. After incubation, the enzyme activity was determined. [15].

RESULTS AND DISCUSSIONS

Optimization of process parameters for induction medium

A new induction medium was prepared by selecting most influencing variables which were optimized using RSM to enhance cell biomass and chondroitinase enzyme production. Based on preliminary experiments, nicotinic acid, chondroitin sulphate, yeast extract and pH were selected as independent variables while K_2HPO_4, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $(NH_4)_2SO_4$ temperature and conditions were kept constant to examine interactions among different variables at different levels. When grown in non-optimized induction medium, *P. penneri* SN5 showed 0.802±0.0067 g of wet cell mass growth per 100 ml of induction medium after 24 h of incubation at 30ºC. Experiments were designed to optimize media constituents and the results obtained were fed into Design Expert 7.0 software and analyzed using analysis of variance (ANOVA) as appropriate tool in used experimental design. Based on the CCD, the experimental levels under each set of condition, the values of wet cell mass were determined. Further these observed values were compared with the correspondent predicted levels (Table 2).

Table 2. Experimental design and results of central composite design for wet cell mass from *P. penneri* SN5.

Run no.	Nicotinic acid in mg	Yeast Extract in mg	Chondroitin Sulphate in mg	pH	Wet Cell Mass in g	
					predicted value	observed value
1	9	95	250	6	0.804	0.802
2	11	95	250	6	0.820	0.817
3	9	105	250	6	0.782	0.785
4	11	105	250	6	0.810	0.817
5	9	95	350	6	0.804	0.798
6	11	95	350	6	0.810	0.816
7	9	105	350	6	0.800	0.795
8	11	105	350	6	0.814	0.810
9	9	95	250	7	0.850	0.848
10	11	95	250	7	0.836	0.830
11	9	105	250	7	0.744	0.739
12	11	105	250	7	0.738	0.748
13	9	95	350	7	0.840	0.843
14	11	95	350	7	0.836	0.827
15	9	105	350	7	0.750	0.757
16	11	105	350	7	0.754	0.751
17	8	100	300	6.5	0.664	0.654
18	12	100	300	6.5	0.672	0.665
19	10	90	300	6.5	1.042	0.988
20	10	110	300	6.5	0.958	0.947
21	10	100	200	6.5	0.830	0.820
22	10	100	400	6.5	0.840	0.828
23	10	100	300	5.5	0.710	0.701
24	10	100	300	7.5	0.672	0.664
25	10	100	300	6.5	0.958	0.942
26	10	100	300	6.5	0.964	0.949
27	10	100	300	6.5	0.966	0.943
28	10	100	300	6.5	0.967	0.943
29	10	100	300	6.5	0.96	0.953
30	10	100	300	6.5	0.958	0.951

Table 3. Regression analysis (ANOVA) for the growth

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	0.2920	14	0.021	798.54	< 0.0001	**significant**
A-Nicotinic acid	0.0002	1	0.000	5.74	0.0300	
B-Yeast Extract	0.0138	1	0.014	529.20	< 0.0001	
C-Chondroitinsulphate	0.0001	1	0.000	3.09	0.0993	
D-pH	0.0012	1	0.001	47.19	< 0.0001	
AB	0.0001	1	0.000	3.10	0.0986	
AC	0.0000	1	0.000	0.04	0.8475	
AD	0.0004	1	0.000	16.88	0.0009	
BC	0.0003	1	0.000	9.80	0.0069	
BD	0.0074	1	0.007	283.13	< 0.0001	
CD	0.0000	1	0.000	0.00	1.0000	
A^2	0.1477	1	0.148	5653.13	< 0.0001	
B^2	0.0025	1	0.003	97.27	< 0.0001	
C^2	0.0274	1	0.027	1050.16	< 0.0001	
D^2	0.1254	1	0.125	4801.84	< 0.0001	
Residual	0.0004	15	0.000			
Lack of Fit	0.0003	10	0.000	1.92	0.2436	***not significant**
Pure Error	0.0001	5	0.000			
Cor Total	0.2924	29				

R-Squared: 0.9987, Adj R-Squared: 0.9974, Pred R-Squared: 0.9935 *not significant lack of fit is necessary for fitting the model.

Table 4. Model fit summary for the response.

Source	R-Squared	PRESS value	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Linear	0.0523	0.3828	0.2771	20	0.0139	856.88	<0.0001	
2FI	0.0802	0.4369	0.2689	14	0.0192	1188.00	<0.0001	
Quadratic	0.9987	0.0019	0.0003	10	0.0000	1.92	0.2436	**Suggested**
Cubic	0.9997	0.0010	0.0000	2	0.0000	0.20	0.8281	**Aliased**
PureError			0.0001	5	0.0000			

The maximum experimental value for optimum wet cell mass was 0.988 g, while the value of predicted response is 1.042 g. At the same time close correlation between the experimental and predicted data indicates the appropriateness of the experimental design. The quality of the model can be checked using various criteria [23] while the regression equation obtained for the optimization of induction media constituents was determined as a function of these variables. ANOVA of the experimental data was carried out, and statistical equation **(C)** representing the optimum wet cell mass yield was obtained as follows:

$$Y = +0.96 + 2.500E-003\,A - 0.024\,B + 1.833E-003C - 7.167E-003\,D + 2.250E-003\,A\,B - 2.500E-004\,A\,C - 5.250E-003AD + 4.000E-003BC - 0.021BD + 0.000CD - 0.073A^2 + 9.625E-003B^2 - 0.032\,C^2 - 0.068D^2 \quad \textbf{(C)}$$

Where Y represents wet cell mass and A, B and C are coded values of nicotinic acid, yeast extract and chondroitin sulphate, respectively. ANOVA result is shown in the quadratic model for response Y (Table 3). According to the present model AD, BC, BD, A^2, B^2, C^2 and D^2 are significant model terms for response Y. Quadratic model was found to be the "best fit model" for optimization of wet cell mass which resulted in increased production of intracellular chondroitinase enzyme. ANOVA for wet cell mass growth (gm) indicated the "*P*-value" to be < 0.0001, which implies the model to be significant. The "lack of fit *F*-value" of 1.92 with *P*-value 0.2436 implies that the lack of fit is insignificant which is very important for fitting the model. There is only a 0.01% chance that a "lack of fit *F*-value" could occur large due to noise [24].

ANOVA indicated the R^2 value of 0.9987 for the response. This confirms an efficient correlation in-between observed and expected values of the quadratic model. The adequate precision which estimates the signal-to-noise ratio of 106.53 (more than 4 is advocated) indicates an adequate signal [23]. Model fitting summary is illustrated for the response (Table 4).

There was variation between highest observed and the predicted value as seen earlier. It was also observed that the highest result was generated due to –α value of variable B, which is beyond the range of defined limit. Hence the experiment was optimized further with executing the necessary criteria in the software. Total thirty four runs were advocated as optimized solutions but by considering the highest desirability and maximum wet cell mass yield, solution no.1 was "suggested" by software and same was selected to repeat the experiment in three replicates (Mean ± SD) to find observed wet cell mass value to compete predicted value 0.9966.

P. penneri SN5 shows low wet cell mass production 0.802±0.0067 g/100ml in non-optimized induction medium, which was enhanced to 1.0847±0.0033 g/100ml in new induction media obtained by RSM model. Figure 1-3 depicts the 3D surface plots and contour graphs, based on the interactions between these variables shows the correlation between increase in the wet cell mass versus concentration of each variable that leads to the high amount of enzyme production. From the plots, it is very easy to understand the actual interactions between two variables for location of optimum levels. Each curve represents extensive combinations of two tests, an elliptical contour plot indicated the interactions between the variables were remarkable while a circular contour plot means otherwise.

The resulting response surface 3D plot Figure 1(A), illustrated the effect of nicotinic acid concentration and pH on the wet cell mass. This result demonstrated that the response surface had a maximum point. The contour graph Figure 1(B), obtained as a function of nicotinic acid concentration versus pH indicated that

wet cell mass production increases up to a certain level with the increase of both nicotinic acid concentration and pH but later it decreases. The interactive effect of both the parameters showed that the optimum cell mass yield depends on a moderate level concentration of nicotinic acid (about 10 mg) and average pH (about 6.5) in induction media.

Figure 2(A) indicates an increase in biomass yield as concentration of yeast extract level increases from 95 mg to 105 mg but no impact of chondroitin sulphate observed though it increased from 250 mg to 350 mg. The contour graph as in Figure 2(B), obtained as a function of yeast extract concentration versus chondroitin sulphate concentration indicated that biomass production increased with the moderate concentration of chondroitin sulphate and with comparative lower concentration of yeast extract. The interactive effect of both the parameters indicated that the production increased gradually with the average levels of chondroitin sulphate with minimum yeast extract.

In Figure 3(A), there was no single impact of yeast extract was observed though it increased from 95 mg to 105 mg. Rather wet cell mass was shown initial increase and further decrease with induced raise in pH from 6.0 to 7.0. The contour graph obtained as a function of yeast extract concentration versus pH level in Figure (3B), showed that the biomass productivity increased with the concentration and interactive effect of both. The combined effect of both the parameters indicated that the production increased gradually with the increase in level of pH from minimum to maximum while with gradual concentration of yeast extract yield is sustained.

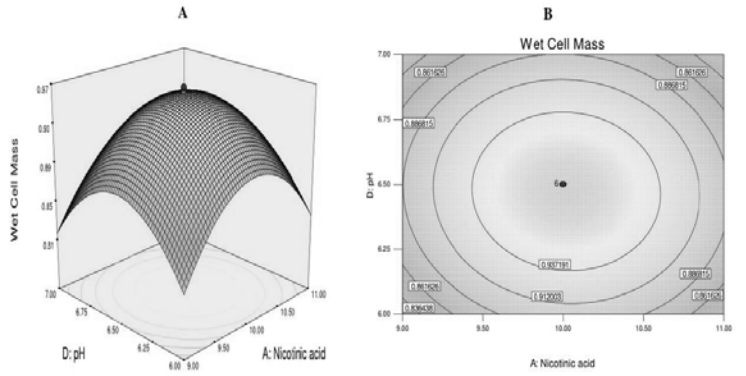

Figure 1: Response surface 3D plots (A) and contour plots (B) indicating interaction between nicotinic acid and pH

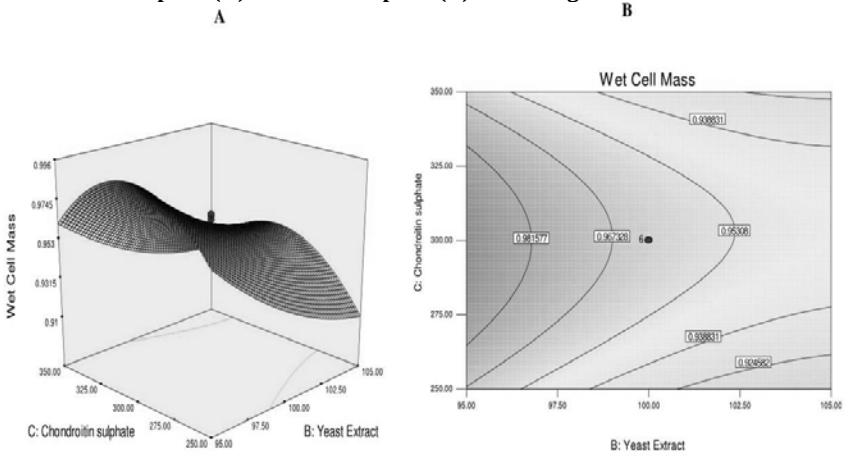

Figure 2: Response surface 3D plots (A) and contour plots (B) indicating interaction between yeast extract and chondroitin sulphate

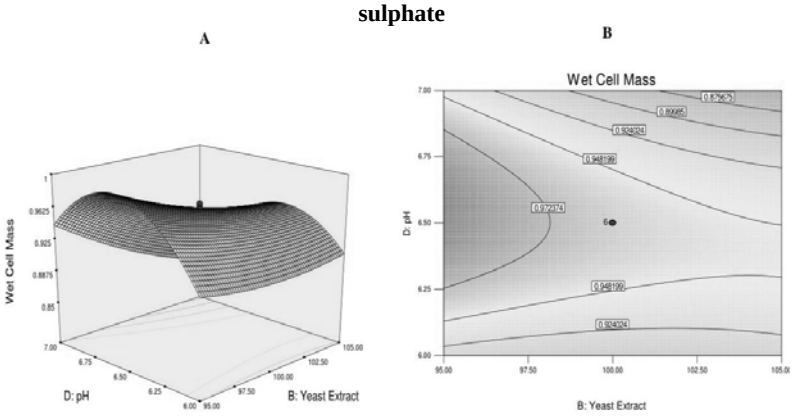

Figure 3: Response surface 3D plots (A) and contour plots (B) indicating interaction between yeast extract and pH

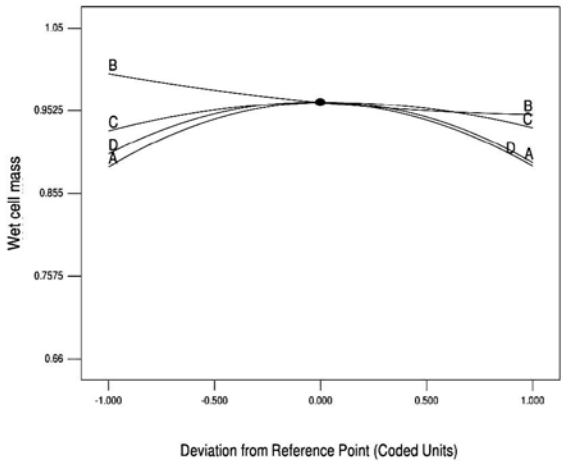

Figure 4: Perturbation curve indicating constituting factors and their effect

Perturbation curve is salient depiction of comparative effect of constituting factors and their effect at a certain point in selected space; real advantage from this plot is in choosing axis and constants in 3D and contour plots. The response is plotted by changing one factor over its range while others kept constant. A sharp slope or curvature indicates the response is sensitive to that particular factor rather flat line shows insensitivity. In case of more than two factors are affecting the response, perturbation curves can determine the most affecting one. X-axis of such plots are made using Delta units or coded units, indicating positions related to coded (-1 to +1) scale. The perturbation plot as in Figure 4, showed that nicotinic acid, pH and chondroitin sulphate having most significant effect on wet cell mass concentration while yeast extract is least but is also worthy to state that interaction of yeast extract with chondroitin sulphate is positive and remarkable in biomass yield.

The maximum biomass productivity of 1.0847±0.0033 g/100ml occurred at a concentration of 10 mg of Nicotinic acid, 95 mg of yeast extract and 298 mg of chondroitin sulphate as substrate with maintaining 6.55 pH, while maintaining K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$, held at 0 coded levels for 100 ml induction media.

Determination of protein content

Protein content was determined in three replicates and the protein concentration plot was plotted at the corresponding absorbance of 660 nm as shown in Figure 5. The protein content in the crude enzyme was determined as 0.89±0.31 mg/ml. It decreased to 0.57±0.17 mg /ml after dialysis and purification by CM-Sepharose column.

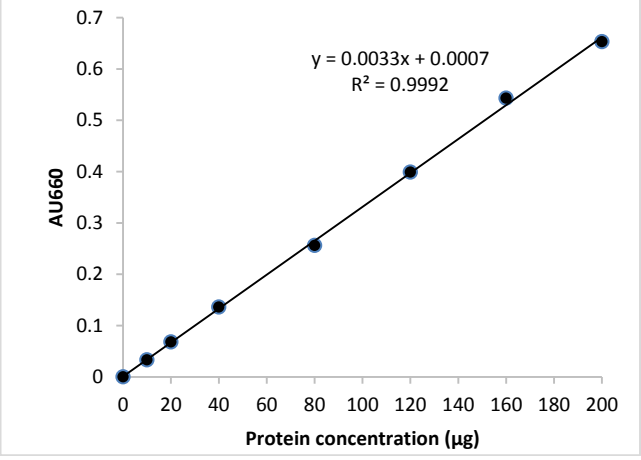

Figure 5: Protein standard curve using bovine serum albumin

Determination of chondroitinase activity

The specific activity of the crude enzyme was found to be 204.43±0.13 U/mg after precipitation of enzyme by 60% ammonium sulphate solution. It increased to 370.38±0.21 U/mg (181.18%) after dialysis and purification by cation exchange column chromatography. The purification fold of the crude enzyme was 1.00, which then increased to 1.81 after dialysis and purification by a cation exchange column. The overall summary of changes in crude and purified chondroitinase enzyme is depicted (Table 5).

Table 5. Changes in parameters of Choroitinase enzyme before and after purification.

Enzyme stage	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Total activity (U)	Specific activity (U/mg)	Purification fold
Crude stage	20	181.94 ± 0.12	0.89± 0.31	3638.87 ± 2.36	204.43 ± 0.13	1
Purified stage	15	211.11 ± 0.12	0.57± 0.17	3166.7 ± 1.77	370.38 ± 0.21	1.81

Values are average of three replicates (Mean ± SD).

Effect of temperature on chondroitinase activity

Chondroitinase enzyme isolated from *P. penneri* SN5 showed almost retained activities at extreme temperatures of 10°C to 40°C. The rapid decline in enzyme activity was observed with further elevation in temperature above 40°C as shown in Figure 6.

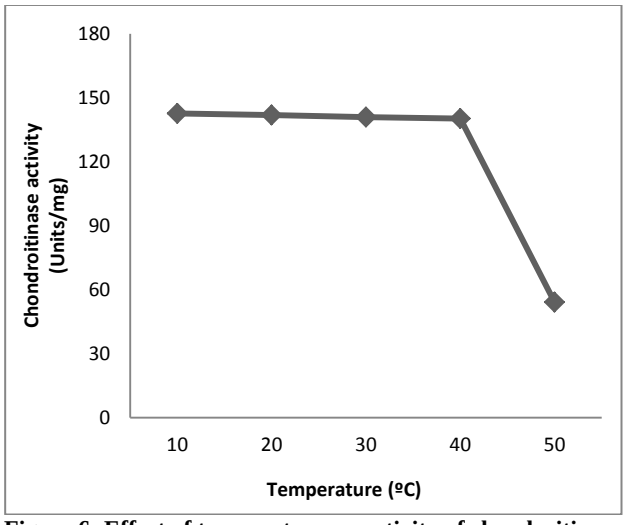

Figure 6: Effect of temperature on activity of chondroitinase enzyme

Effect of pH on chondroitinase activity

The isolated chondroitinase enzyme from *P. penneri* SN5 was showed the increase in its residual activity with raising of pH from 2 to 8. An optimum activity was recorded at a pH 8.0 as depicted in Figure 7. It was observed that the enzyme activity was sharply decreased when the pH was further raised from 8 to 10.

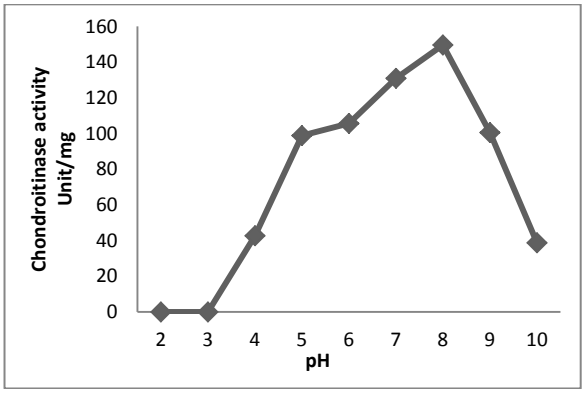

Figure 7: Effect of pH on activity of chondroitinase enzyme

CONCLUSION

A new strain of *Proteus penneri* SN5 was found to be a potential producer of chondroitinase enzyme. In this study, result of a bioprocess was optimized for further production process in *P. penneri* SN5. Central composite design was applied to find out optimum values of significant response factors, which resulted in the wet cell mass index in optimized induction media for therapeutic chondroitinase enzyme production. It is interesting to report that among *Proteus* genus *P. vulgaris* is only the strain was known for chondroitinase enzyme production but *P. penneri* though it is known foremost similar identities [24] to *P. vulgaris* including chondroitinase enzyme producing ability, no attempts were made for optimization and production of chondroitinase enzyme till date. By this study, it is tried to isolate and purify the chondroitinase enzyme at lab scale with low cost where the industrial needs are satisfied. The progress in the chondroitinase research will facilitate the application of this enzyme in fibro-proliferative diseases including in neuronal disc herniation due to spinal injury [25] and thus shall have a profound influence on human health.

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