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Molecular Identification and Optimization of Amylase Producing *Bacillus gingshengii* SNB12 Using Response Surface Methodology

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ABSTRACT

Among different types of enzymes obtained from microbial sources, amylases are the most widely used in industries as well as on commercial basis. Amylase production from bacteria is economical as the enzyme production rate is higher as compared to other microorganism. The aim of the current study was to investigate the genotypic analysis of starch degrading bacteria from mangrove soil. The strain was identified as catalase and gram positive bacteria. On the basis of phylogenetic analysis using the 16S rRNA, this strain was identified as *Bacillus gingshengii* SNB12. α-Amylase production by *Bacillus gingshengii* SNB12 was cultivated in medium containing 1% soluble starch as a carbon source. The process parameters such as incubation day, percentage of inoculum and starch were optimized for maximum amylase production using central composite design (CCD) of response surface methodology (RSM). Total 20 experiments were carried out in conical flask and a three dimensional response surface was generated to determine the effect of process variables on amylase production. Solid state fermentation (SSF) was performed using 5.0 g of Sugarcane bagasse (5g) inoculated with $1x10^7$spores. Incubation day and percentage of starch were the most significant ($p<0.05$) parameter and the maximum interaction occurred between incubation day and percentage of inoculum. The results of the study indicated that amylase is maximized (274 U/ml) at optimized levels of 2, 2.5 and 3 days for percentage of inoculum, starch and incubation periods, respectively.

Keywords: Amylase, Phylogenetic analysis, *Baciliius sps*, Response surface methodology

INTRODUCTION

The starch hydrolytic amylases (α-amylase, β-amylase and glucoamylase) are one of the most extensively used enzymes in present-day biotechnology. Amylases hydrolyze α-1,4 glycosidic linkages of starch to yield dextrin and different monomeric products. Microbial amylases are of immense value in industries due to bulk enzyme production and easy way of genetic manipulation (Pranay *et al*., 2019). Although amylases are produced from different sources such as microorganisms, plants and animals microbial amylase are most suitable for various industrial application. Amylases obtained from bacterial species are known for greater stability, short growth period, high productivity, reduced cost of production and easy manipulation of bacterial genes. Increasing industrial demand for microbial amylases has been observed due to their specificity of reaction, mild conditions prerequisite for the reaction, and less energy consumption than the conventional non-enzymatic chemical methods.

Among various microbial sources *Bacillus sp*. is ideal for industrial production of enzymes due to attributes such as short fermentation cycle, consistency, efficient enzyme activity under stress conditions, and safe and easy handling (Souza and Magalhaes, 2014). Bacteria and fungi tend to secrete amylases outside the cells to perform extra cellular digestion of starch into sugars. Extensive application of amylase in various industrial sectors such as food, starch liquefaction, saccharification, brewing, detergent, paper, textile and distilling industries, has brought about a greater attention for the increase in the indigenous production of α-amylase. (Gupta *et al*.,2003). *Bacillus* is a common bacterial source for industrial amylase production. However, different strains have different optimal growth conditions and enzymatic production profile. Reportedly, *Bacillus* strains have been extensively used in industrials to produce α-amylase which includes *B. amyloliquefaciens, B. subtilis B. licheniformis, B. stearothermophilus, and B. megaterium*

Solid-state fermentation (SSF) uses solid substrates at low moisture levels and requires suitable agroindustrial materials as solid substrate (Hamrouni *et al*., 2019). Various agroindustrial waste used as susbtrate for SSF which includes sunflower cake olive, (Kachrimanidou *et al*., 2013), sugarcane bagasse (Leite *et al*., 2019) fruit's peels and pulps (Papadaki *et al*., 2019) and cereal brans (Soccol *et al*., 2017). In this process, microorganisms grow and produce a wide variety of products such as mushrooms (Postemsky *et al*., 2017) microbial oil (Tsakona *et al*., 2014), preservatives like fumaric acid (Papadaki *et al*., 2018) and enzymes (Haque et al., 2016) reducing the cost of production.

Identification of the organisms was done on the basis of 16S rDNA based molecular technique. The sequence alignment was carried out using ClustalX (Larkin et al., 2007)) which is a version with graphical user interface in Clustal series for multiple sequence alignment. The phylogenetic tree was constructed using ClustalX with neighbor-joining method maximum likelihood, and presented using MEGA X version (Kumar *et al*., 2018). The phenotypic characterization and 16S rRNA indicated that bacterial cultures belongs to genera Bacillus. The amylase production from different *Bacillus sp*. shows a great deal of variation because the production of amylase depends on the composition of medium and other physical parameters. The present work focus on the molecular identification and optimization of bacterial strain amylase production using SSF.

MATERIALS AND METHODS

Collection and isolation of soil sample

Sample collection Soil samples were randomly collected 15 cm below the soil surface and kept in polystyrene bags from various mangrove areas near Astamudi lake, Kollam District, Keral, India Isolation of bacteria was performed by serial dilution in which 1 g of the soil sample were weighed and suspended into 9ml of sterile distilled water taken in test tube and shaken vigorously with the help of a vortex machine (10^{-1}). Similarly, dilutions were carried out up to $10^{-6.}$ All the dilutions were plated on Nutrient agar medium (Himedia M-001) and incubated at room temperature for 3days.

Bacterial inoculums preparation

50 ml of nutrient broth (13 g/l at pH 7.4 ± 0.2) was prepared and sterilized in an autoclave (NatSteel (Big), India) at 15 lbs/in 2 pressure, 121°C for 20 min. Freshly grown 1ml of the inoculum were aseptically transferred to media and incubated at 37°C overnight at 120 rpm in a incubator shaker (Lab Tech , India).

Solid Substrate preparation and Pretreament

Among different agricultural residues, Sugarcane bagasse were used as solid substrate (support and nutrient source) for SSF. Due to the water content, the residues were sun-dried for 2 days and then oven dried at 80°C for 24 h. The dried sugarcane bagasse were stored in air-tight container until required. The moisture content of the solid substrate was estimated by drying 5 g of substrate to a constant weight at 70 °C for 24 h and the dry weight was recorded. To fix the initial moisture content of the solid medium, 5 g of substrates was soaked with 5 mL inoculums and 4mL fermentation media. After soaking, the solid substrate was again dried as described above and the percent moisture content was calculated using Equation as follows

$$Initial\ moisture\ content\ (\%) = \frac{W_{Final} - W_{Initial}}{W_{Initial}} \times 100\%$$

where W_{Final} is the weight of the dried solid substrate after soaking and $W_{Initial}$ is the weight of dried solid substrate before soaking. From the above procedure, it was found that the initial moisture content of different solid substrate.

Quantitative screening for amylase

The amylase activity of the strain was demonstrated on starch-casein agar plate containing 1% soluble starch after 4 days of incubation at 30°C, the plates were stained with Gram's iodine solution (0.2% I_2 and 2% KI). α-Amylase production in solid state fermentation (SSF) was carried out in 250mL Erlenmeyer flask using basal medium containing 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 5.0 g NH_4Cl, 0.5 g NaCl, 0.15 g $CaCl_2$, 0.25 g $MgSO_4$ $7H_2O$, 0.2 g Casein hydrolysate, 0.10g Yeast extract, 10 g Starch per liter of distilled water at pH 7.0.

Enzyme production in SSF

The SSF process was carried out in 250 ml Erlenmeyer flask using Sugarcane bagasse. After proper agitation of the substrate with 4ml of the media, it was autoclaved at 121°C for 15 min. Later it was allowed to cool and incubated with 1ml of 24 h old bacterial culture. Subsequently, incubation was carried out at 37 °C for 7 days. The SSF media flask was shaken after every 24 h for uniform mixing up of the substrate and inoculum.

Enzyme extraction

The fermented mass was mixed with 0.1M phosphate buffer (pH 7.0) to a volume of 100 mL for each flask and agitated at 180 rpm for 30 min. The slurry thus obtained was squeezed through a cheese cloth, followed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant was collected in vials and stored at 4°C for further analysis.

α-Amylase Assay

The α-amylase activity was estimated by analysing reducing sugar released during hydrolysis of 1.0% (w/v) starch in 0.1M phosphate buffer (pH 7.0) by enzyme (cell-free supernatant) incubated at 80°C for 10min.The amount of reducing sugar level released in the mixture was determined by the dinitrosalicylic acid (DNS) method Miller. Absorbance at 550 nm was recorded by using UV-visible spectrophotometer (UV-1700 Pharmaspec Shimadzu) and activity was calculated from a standard curve using maltose as the standard. One unit (U) of enzyme activity was defined as the amount of enzyme required for the liberation of 1 μmol reducing sugar as maltose per minute under standard assay conditions. All experiments were carried out in triplicate and the data presented are average values.

Optimization of incubation period, percentage of inoculums, Starch and maltose by applying RSM

The characterization of three different factors for amylase production was optimized by applying the RSM. The statistical approach using the Central Composite Design (CCD) developed by 'Design Expert 10.0 software StatEase, Inc. Minneapolis, USA, was used to generate and analyze the 24 experimental design. The statistical model was obtained with three independent variables [incubation period (A), Percentage of inoculum (B) and Starch (C). Each factor in this design was studied at four different levels (Table 1). All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were used. The full experimental plan with respect to their values in coded form is shown in Table 2. After the completion of experiments, the average of amylase production was taken as the dependent variable or response.

Statistical analysis and modeling

The data obtained from the RSM on α-amylase production was finally subjected to the analysis of variance (ANOVA). The results of RSM were used to fit a second order polynomial equation (1) as it represented the behavior of such a system more appropriately.

$$\begin{aligned} Y= \beta0 + \beta1A + \beta2\ B + \beta3\ C + \beta1\ \beta1\ A2 + \beta2\ \beta2\ B2 \\ + \beta3\ \beta3C2 + \beta1\ \beta2\ AB + \beta1\ \beta3\ AC + \beta2\ \beta3\ BC \qquad (1) \end{aligned}$$

Where Y was response variable, $\beta0$ was intercept, $\beta1$, $\beta2$ and $\beta3$ were linear coefficients, $\beta1,1$, $\beta2,2$ and $\beta3,3$ were squared coefficient, $\beta1,2$, $\beta1,3$ and $\beta2,3$ were interaction coefficient and A, B, C, A2, B2, C2, AB, AC and BC were the level of independent. The Fisher's test value was used to determine the statistical significance of the model equation and and the production of variance explained by the model was specified by the multiple coefficient of determination, R squired (R2) value. Design Expert (ver, 10.0; STATEASE INC; Minneapolis, MN, USA) was used in this investigation.

Coded Independent variables Factor Levels

Table 1 -Range of the values for the response surface methodology.

Independent varibles	Coded factor level			
	-α	-1	+1	+α
Incubation day	1.31	2	4	4.681
Percentage of inoculum	0.02	1	4	5.02
Starch	0.659	1	2	0.659

Effect of incubation period on enzyme production

The inoculum was prepared in soluble amylase production medium by transferring a loop full of bacterial strain from a slant and was incubated at room temperature for 24 h in an orbital incubator shaker at 120 rpm. (Orbiteck , India). Sugarcane biagasse (5 g) was taken in conical flask, moistened with 9 ml of amylase medium to provide 60% moisture holding capacity (MHC) and the contents were mixed thoroughly. The bottles were autoclaved at 15 lb pressure for 30 min. After cooling at room temperature, (30°C) these were inoculated with 1% (v/w) inoculum and incubated under static condition at room temperature for 7 days. Triplicate bottles were maintained for each treatment. The contents in the bottle were periodically mixed by gentle tapping. At interval of 24 h, the enzyme were extracted twice with 25ml of 0.1M Phosphate buffer. Initially the substrate were soaked in Phosphate buffer for 30 min at 30°C and kept in a rotatory shaker at 150 rpm. The slurry thus obtained were squeezed through a wet cheese cloths. The pooled enzyme extract were centrifuged at 10000 rpm for 20 min in refrigerated centrifuge and the clear supernatant was further stored for enzyme assay.

Effect of percentage of inoculum

The influence of percentage of inoculum on the enzyme titer was evaluated by varying the inoculum percentage of the substrate from 1 to 5%. Different percentage of inoculum were incubated for 7 days at room temperature.

Effect of Starch concentration

The effect of starch concentration on the enzyme titer was evaluated by varying the starch concentration from 1 to 5% Different percentage of starch were incubated with the sugarcane for 7 days at static condition in room temperature.

Molecular identification using 16*S* rRNA Gene Sequencing and Phylogenetic Analysis

The total genomic DNA was extracted using NucleoSpin® Tissue Kit (Macherey-Nagel). 16S rDNA sequence were amplified from genomic DNA using universal primer (Integrated DNA Technology,India) 16S-RS-F-5-CAGGCCTAACACATGCAAGTC-3 and 16S-RS-R 5-GGGCGGWGTGTACAAGGC-3 in a PCR thermal cycler (Nubel *et al.*, 1996). PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer, 0.2mM each dNTPs, 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and 2µl of template DNA. Amplification of DNA for 20 µL reaction was carried out under the following condition. The PCR conditions were set for 35 cycles with initial denaturation at 95°C for 5 min then final denaturation of 95°C for 30 sec, annealing at 60ºC for 40 sec and final extension at 72ºC for 60 sec using Thermal cycler (Gene Amp PCR System 9700, Applied Biosystems). The PCR products were detected on 0.8% agarose gel. The PCR products were sent to for sequencing. The 16S rRNA gene sequences were compared with other 16S rRNA gene sequences available in GenBank by using the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned with similar sequences by using CLUSTX program. The phylogenetic trees were constructed by neighbor-joining statistical method using MEGA X (https://www.megasoftware.net */dload_win_gui)*. In order to determine the stability of phylogenetic tree, the sequence data were sampled 1000 times for bootstrap analysis using MEGA X with 50% cut-off (Kumar *et al*., 2018).

RESULTS AND DISCUSSION

Isolation and Screening of amylase producing Bacteria.

A total of 20 amylase producing bacterial strains were successfully isolated from different locations of mangrove soil. Among the 20 strains isolated, 5 strains found to be amylase producers based on their zone formation on starch agar media (Fig1).The total bacterial population (CFU/ml) was observed at 10^{-5} dilution for all the tested soil samples. The population was high of 2.97×10^{7}CFU/ml in the soil sample (SNB12) followed by the soil sample (SNB6) with 1.98×10^{7}CFU/ml. The least bacterial population was found in (SNB8) soil sample, where only1.3×10^{7}CFU/ml was observed. Among the isolates further screened for its capability to degrade starch only five isolates were selected based on the zone diameter listed in Table 1. Among twenty strains isolated, SNB12 were selected for further optimization and phylogenetic analysis based on the hydrolyzing zones on agar plates containing starch substrate (Fig 1).

Table 1: The hydrolyzing zone diameter of isolates

Screening of amylase producing bacteria		
Isolates	**Screening test**	**Diameter zone (mm)**
SNB1	+	3.6
SNB3	+	2.5
SNB7	+	1.5
SNB8	+	3.1
SNB12	+	4.2

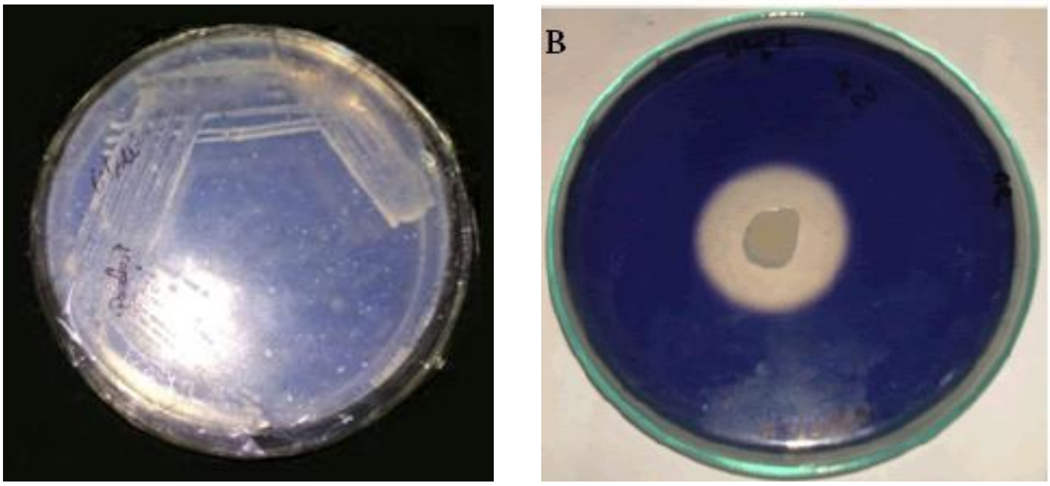

Fig 1 A: Initial screening of bacterial strain in nutrient agar plate ; B: Hydrolyzing zones produced by bacterial strains on starch agar plates after flooded with iodine solution.

Optimization using Response surface methodology

The Central Composite design was employed to study the effect of three independent fermentation variables (incubation period, Percentage of inoculum and Starch) along with the mean predicted and observed responses are presented in Table 2. In the present work, experiments were planned to obtain a quadratic model consisting of 2^3 trials. The plan includes 20 experiments and two levels of concentration for each of the component. In order to analyze the combined effect of these components, experiments were performed at different combinations. Table 4 summarizes the Central Composite experimental plan along with the predicted and observed response for each individual experiment. It shows the production of alpha-amylase (mg/ml) corresponding to combined effect of all three components in the specified ranges. The regression equations obtained after the ANOVA gave the level of α-amylase production as a function of the initial values of incubation period, percentage of inoculum and temperature. The final response equation that represented a suitable model for α-amylase production was as below:

$$Y=279.23-19.09A-10.13B+4.44C+3.40AB-0.52AC$$
$$+3.95BC-97.44A^2-103.65XB^2+4.35C^2$$

Where Y was enzyme production, A was incubation period (h), B was percentage of inoculum (%) and C was starch (mg/ml).

Table 2. Experimental design and result of CCD of response surface methodology

Std	Run	Incubation day	Percentage of inoculum	Starch	Amylase (mg/ml)	Predicted value
9	1	1.68	2.5	2.25	106.3	135.59
8	2	4	4	2.5	58.2	64.54
7	3	2	4	2.5	107.7	96.97
23	4	3	2.5	2.25	279	279.93
2	5	4	1	2	64.9	70.16
12	6	3	4.4	2.25	84	86.38
13	7	3	2.5	1.92	276.9	280.91
5	8	2	1	2.5	127.5	116.13
15	9	3	2.5	2.25	269.8	279.23
6	10	4	1	2.5	68.3	70.10
14	11	3	2.5	2.5	284	292.61
17	12	3	2.5	2.25	281.9	279.23
22	13	3	2.5	2.25	286.3	279.23
4	14	4	4	2	42.9	48.80
10	15	4.3	2.5	2.25	102	85.34
16	16	3	2.5	2.25	286.3	279.23
20	17	3	2.5	2.25	279.4	279.23
18	18	3	2.5	2.25	284.9	274.23
21	19	3	2.5	2.25	286.54	280.16
1	20	2	1	2	125.9	114.10
24	21	3	2.5	2.25	297.7	279.23
11	22	3	0.525889	2.25	102.8	113.04
19	23	3	2.5	2.25	273.9	279.23
3	24	2	4	2	86.4	79.13

The analysis of variance (partial sum of squares) were shown in Table 3. The Model F-value of 159.28 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 11.68 implies the Lack of Fit is significant. There is only a 0.10% chance that a "Lack of Fit F-value" this large could occur due to noise.

The Response surface was generated by plotting the response (α-amylase production) on the z-axis against any two independent variables while keeping the other independent variable at their zero level. Therefore, three response surfaces were obtained by considering all the possible combinations.

Table 3. Analysis of Variance (ANOVA) of alpha amylase production depicted from CCD

Source	Sum of squares	df	Mean square	F value	P- value	significant
Model	2.250E+005	9	25003.63	159.28	<0.0001	significant
A-Incubation day	4178.20	1	4178.20	26.62	0.0001	significant
B- % of inoculum	1176.63	1	1176.63	7.50	0.0160	significant
C- Starch	226.39	1	226.39	1.44	0.2497	not significant
AB	92.48	1	92.48	0.59	0.4555	not significant
AC	2.20	1	2.20	0.014	0.9073	not significant
BC	124.82	1	124.82	0.80	0.3876	not significant
A2	69982.59	1	69982.59	445.81	0.0001	significant
B2	79181.65	1	79181.65	504.41	0.0001	significant
C2	139.23	1	139.23	0.89	0.3623	
Residual	2197.71	14	156.98			
Lack of fit	1904.28	5	380.86	11.68	0.0010	significant
Cor Total	2.272E+005	23				

Std.Dev	12.53	R-squared	0.9903
Mean	182.26	Adj R-squared	0.9841
C.V%	6.76	Pred R-squared	0.9369
PRESS	14334.46	Adeq Precision	30.146
-2 Log Likelihood	176.52	BIC	208.30
		AICc	213.44

An adequate precision of 30.146 for α- amylase production was recorded. The predicted R2 of 0.9365 was in reasonable agreement with the adjusted R2 of 0.9841. This indicated a good agreement between the experimental and predicted value for α-amylase production. The model F- value of 159.28 and values of prob> F (< 0.05) indicated that the model terms were significant. In analyzing the effect of variables, normally the 3-D contour plots were used. 3-D contour plots represent the relationship of response surface function of two variables; meanwhile another variable is maintained at zero level. The coordinates of the central point within the highest contour levels in these figures represent the optimum condition and concentrations of respective parameters. The circular shape of the curve indicates that no interaction occurred, whereas the elliptical shape indicated good variation of the two variables.

Effect of amylase production on incubation day and inoculum

To investigate the interaction and to visualize the effects of the two factors on amylase activity, a graphical representation of RSM is presented in Fig. 2 depicts three dimensional diagram of calculated response surface from the interaction between incubation day and percentage of inoculum while keeping the other variable (Starch) at ‘0’ level. The response surface obtained in this study was convex in nature, which suggested that the optimum conditions were well defined. A gradual increase in α-amylase production was observed when incubation period was increased up to third day of incubation, and there after, decline in enzyme production was notified. it can be observed that the enzyme activity was increased upon the percentage of inoculum at the range of 1 to 4%. Further increase in the percentage of inoculum and prolonged incubation day have a positive effect on the response until certain optimum value, whereas extremely high values of these variables will inhibit amylase production.

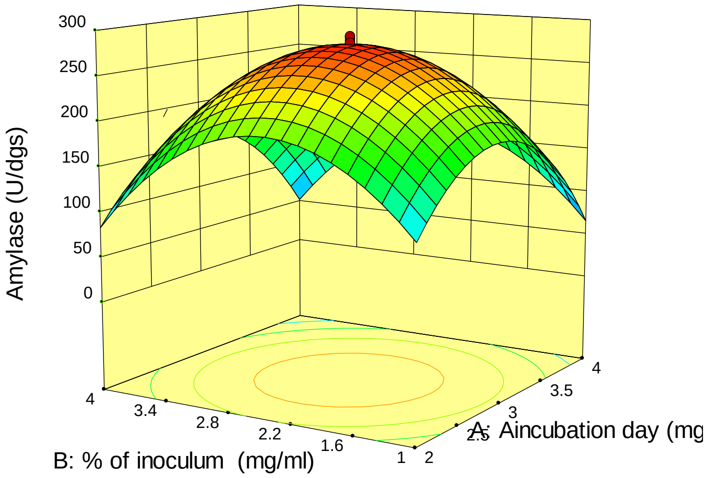

Figure 2: Response surface plot showing the effect of incubation day and percentage of inoculum.

Effect of amylase production on incubation day and starch

In figure 3, the activity of alpha amylase increased upon the incubation day and reached maximum on the third day. However, further increase in the incubation day had no effect in the enzyme production. The concentration of starch from 2 to 2.5% resulted no increase in the enzyme production.

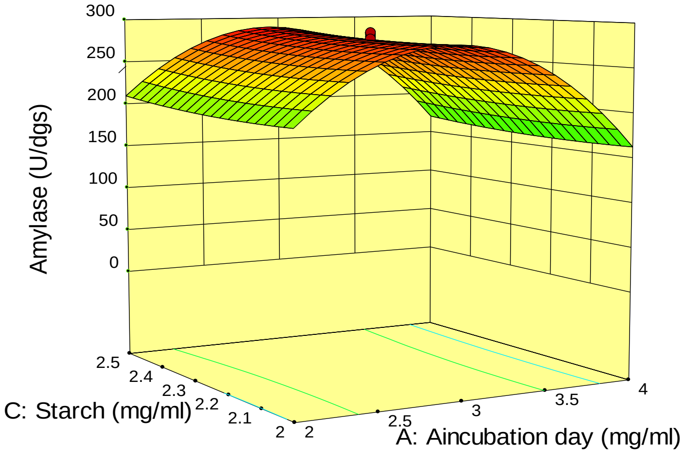

Figure 3: Response surface plot showing the effect of incubation day and starch

Effect of amylase production on starch and inoculum

When the percentage of inoculum was increased, increase in amylase production were detected and reached optimum on the third day of incubation. later on increase on the incubation day showed a gradual decrease in enzyme production Whereas, starch concentration of 2 to 2.5% showed no increase in the enzyme production. The details are shown in fig 4.

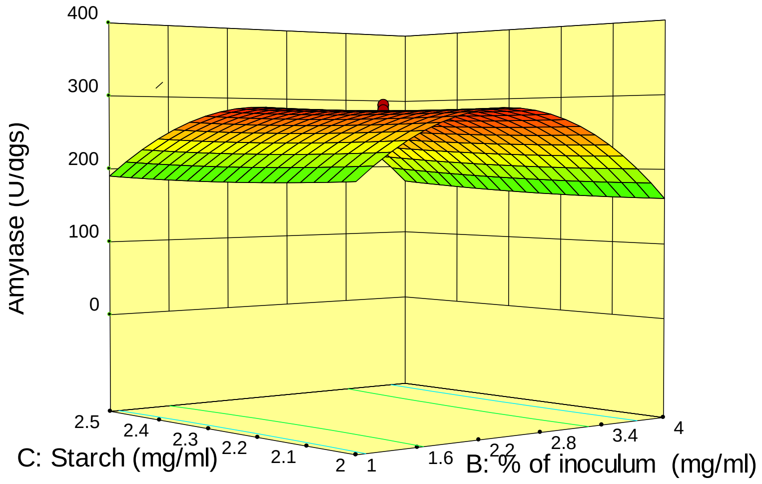

Figure 4: Response surface plot showing the effect of percentage of inoculum and Starch

Validation of model was carried out under the conditions predicted by the response surface model. The experimental values were very close to the predicted values;

hence, the model was successfully validated. The validation of the statistical model and regression equation was performed by taking incubation day (third day), Percentage of inoculum (2.5%) and starch (2%) in the experiment. The predicted response for α-amylase production was 292.21 mg/ml, while the actual (experimental) response was 284.67 mg/ml, thus proving the validity.

Molecular identification of keratinase producing bacteria

The amylase producing bacteria were recovered from starch agar media. The bacterial stain was further subjected to molecular identification procedure which includes extraction of total DNA, amplification by 16S rDNA primer, finally the comparison of phylogenetic sequence.

DNA extraction

Total genomic DNA was extracted from SNB12 Fig 5A. For further identification the amplification of the genomic DNA was performed using 16S rDNA primer (Fig 5B). Later, gene sequencing and construction of phylogenetic tree was done for the species identification.

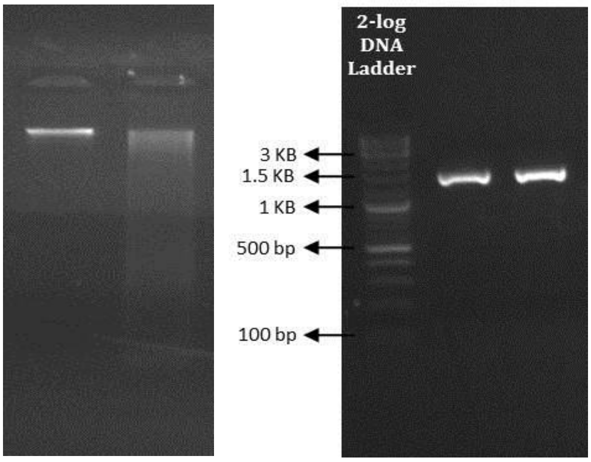

Fig 5. A: Genomic DNA from bacterial strain using DNA isolation kit loaded in 0.8% agarose gel

B: PCR amplification of 16s rDNA fragment from bacterial sample.

Sequence Analysis

In this work, we have provided the molecular identification of bacteria on the basis of 16S rDNA analysis. The results of the forward and reverse primers were aligned together in Bioedit tool to retrieve the complete aligned sequences. The NCBI BLAST was performed to confirm identity of specimens (Altschul, *et al.*1990). The 16S rRNA gene sequence was compared to the Gen-bank of database using the

BLAST. The 16S rRNA gene sequence of the isolate SNP1 showed high levels of sequence similarity with members of the genus *Bacillus.* The 'BLASTn' tool was used for sequence assignment against NCBI database and highest-scoring hit from each query is taken for the bacterial identification. The Blast result predicted based on percent match given bacterial sequence belongs to *Bacillus* genus and alignment of gene sequence with 10 closely related gene sequence was performed and phylogenetic tree was constructed using MEGA X (Fig 6.) The bacterial strain was identified as *Bacillus qingshengii*

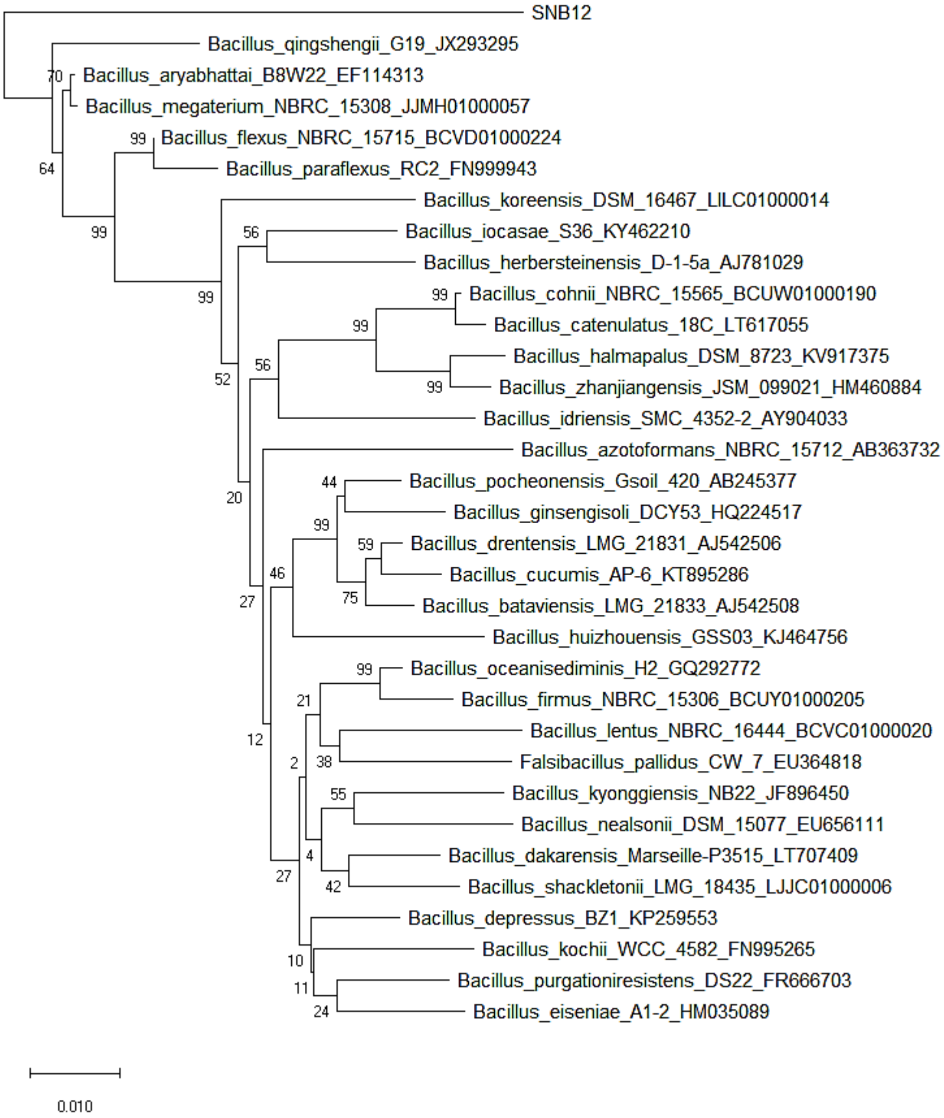

Fig 6: Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.53056807 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). and are in the units of the number of base substitutions per site. The analysis involved 33 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1536 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2014).

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Conflict of interests

The authors declare that they have no competing interests.

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