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Isolation, Screening and Identification of Actinobacteria with Uricase Activity: Statistical Optimization of Fermentation Conditions for Improved Production of Uricase by *Streptomyces rochei* NEAE-25

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ABSTRACT

One hundred and thirty actinomycetes soil-isolates were screened for their uricase activity. The most promising isolate, strain NEAE-25, was selected and identified on the basis of morphological, cultural, physiological and biochemical properties, together with 16S rRNA sequence as *Streptomyces rochei* NEAE-25 and the sequencing product was deposited in the database of GenBank under accession number HQ889312. The optimization of different process parameters for uricase production by *Streptomyces rochei* NEAE-25 and its validation using Plackett-Burman experimental design and response surface methodology was carried out during the present study. Fifteen variables were screened using Plackett-Burman experimental design. The most significant positive independent variables affecting enzyme production (incubation time, medium volume and uric acid concentration) were further optimized by central composite design. The maximum uricase production by *Streptomyces rochei* NEAE-25 after central composite design was 47.49 U mL^{-1} with a three-fold increase as compared to the unoptimized medium (16.1 U mL^{-1}).

Key words: *Streptomyces*, uricase, optimization, Plackett-Burman experimental design, response surface methodology, 16S rRNA, scanning electron microscope

INTRODUCTION

Uric acid is the end product of purine metabolism in the human body and is excreted by the kidney out of the body. It is well authenticated that overproduction and accumulation of uric acid over than the normal value (hyperuricemia) in humans blood results in renal failure (Capasso *et al*., 2005) and may cause gout disease (Nakagawa *et al*., 2006), idiopathic calcium urate nephrolithiasis (Masseoud *et al*., 2005) and it was also reported that leukemia in children associated with an elevated uric acid level (Larsen and Loghman-Adham, 1996), toxemia of pregnancy (Kelly and Pelella, 1987). Uricase (urate oxidase, urate oxygen oxidoreductase, EC 1.7.3.3) is an enzyme that catalyzes the enzymatic oxidation of uric acid to allantoin, carbon dioxide and hydrogen peroxide (Brogard *et al*., 1972) which is more soluble and easily to be excreted than the uric acid:

$$\text{Uric acid} + H_2O + O_2 \xrightarrow{\text{Uricase}} \text{Allantoin} + H_2O_2 + CO_2$$

The application for uricase as a diagnostic reagent for the determination of uric acid in biological fluids such as blood and urine was identified as the first important application of uricase in clinical biochemistry (Adamek *et al*., 1989). Uricase

has been widely used for enzymatic determination of uric acid in routine clinical analysis by coupling it with a 4-amino-antipyrine-peroxidase system (Capasso *et al*., 2005; Gochman and Schmitz, 1971). This enzyme can be also used therapeutically as a protein drug to reduce toxic urate accumulation (Colloc'h *et al*., 1997). Immobilized uricase can be used as a uric acid biosensor (Arslan, 2008). Uricase is also used as an additive in commercial formulations of hair coloring agents (Nakagawa *et al*., 1995). Uricase is absent in humans but is widely present in most vertebrates.

Some microorganisms such as *Gliocladium viride* (Nanda *et al*., 2012), *Pseudomonas putida* (Poovizh *et al*., 2014) and *Nocardia farcinica* (Ishikawa *et al*., 2004) have been used to produce uricase. Although uricase has been produced using several microbial sources, due to its increasing importance in treatment and in diagnosis, new sources of uricase are sought aiming to produce better yield of the enzyme (Yazdi *et al*., 2006). The largest and most important genus in the order actinomycetales are *Streptomyces*. It comprises up to 90% of actinomycetes isolated from the soil samples, it is prolific producers of bioactive compounds such as antibiotics and enzymes which have important applications both in medicine and agriculture (Demain and Sanchez, 2009; El-Naggar and Abdelwahed, 2012, 2014; El-Naggar *et al*., 2014).

The composition of culture medium strongly influences the growth and enzyme production by microorganisms, thus optimization of cultural parameters and medium components can significantly affect product concentration, yield and the ease and cost of downstream product separation (Wang *et al*., 2008). The optimizations of cultural parameters and medium components have been performed traditionally using one-factor-at-a time method. The drawbacks of this method are that it ignores the combined interactions among different variables; it is time consuming especially for a large number of variables, laborious and it is expensive (Bandaru *et al*., 2006). Therefore, in recent years, full factorial or Plackett-Burman design and response surface methodology were used to search the factors rapidly from a multivariable system (Aghaie-Khouzani *et al*., 2012). Response surface methodology has eliminated the drawbacks of traditional optimization methods, it has the advantage of taking into account the interaction between the nutrients and is less time consuming (Deepak *et al*., 2008; Banik *et al*., 2007).

The aim of the present study was to screen some microbial isolates for uricase production, to identify the most potent producer isolate using a combination of phenotypic and genotypic characteristics, Plackett-Burman design was used in the first optimization step to screen the important variables that influence uricase production by *Streptomyces rochei* NEAE-25. The factors that had significant effects on uricase production were further optimized using a central composite design in the second step.

MATERIALS AND METHODS

Microorganisms and cultural conditions: *Streptomyces* spp. used in this study were isolated from various soil samples collected from different localities of Egypt. Actinomycetes had been isolated from the soil using standard dilution plate method procedure on Petri plates containing starch nitrate agar medium of the following composition (g L^{-1}): Starch, 20; KNO_3, 2; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $CaCO_3$, 3; $FeSO_4.7H_2O$, 0.01; agar, 20 and distilled water up to 1 L; then plates were incubated for a period of 7 days at 30°C. Nystatin (50 µg mL^{-1}) was incorporated as an antifungal agent to minimize fungal contamination. The actinomycetes strains predominant on media were picked out, purified and maintained on starch-nitrate agar slants. These strains were stored as spore suspensions in 20% (v/v) glycerol at -20°C for subsequent investigation.

Primary and secondary screening of uricase production: The culture medium for screening consists of (g L^{-1}): Uric acid, 5.0 g; glycerol, 30.0 g; NaCl, 5.0 g; K_2HPO_4, 1.0 g; $MgSO_4$. 7 H_2O, 0.2 g; $CaCl_2$, 0.1 g; distilled water up to 1 L; pH 7. For plates 20.0 g agar was added (Azab *et al*., 2005). Preliminary screening for uricase production was done by conventional spot inoculation of pure actinomycetes strains on agar medium and incubated at 30°C for seven days. Uricase production by the microorganisms is indicated by the appearance of clear zone around the colonies.

The strains which forming bigger clear zones in a shorter time were selected for subsequent screening under submerged fermentation conditions. Fifty milliliter of fermentation medium were dispensed in 250 mL Erlenmeyer conical flasks, sterilized and inoculated. The fermentation media were incubated for at 30°C in a rotatory incubating shaker (200 rpm). The enzyme production was measured after 7 days. The strain which showed the most promising result was selected for further experiments.

Inoculum preparation: Two hundred fifty milliliter Erlenmeyer flasks containing 50 mL of yeast-malt extract broth (malt extract 1%; dextrose 0.4%; yeast extract 0.4%; pH 7.0) were inoculated with three disks of 9 mm diameter taken from the 7 days old stock culture grown starch nitrate agar medium. The flasks were incubated for 48 h in a rotatory incubator shaker at 30°C and 200 rpm and were used as inoculum for subsequent experiments.

Uricase assay: The principle of enzyme measurement was as follows: uricase can catalyse the oxidation of uric acid to form allantoin, carbon dioxide and hydrogen peroxide which is then analyzed by the oxidative coupling of 4-aminoantipyrine, phenol and peroxidase as chromogens. Uricase activity was measured by incubating 300 µL enzyme solution with a

mixture of 400 µL sodium borate buffer (pH 8.5, 0.1 M) containing 2 mM uric acid, 150 µL 4-aminoantipyrine (30 mM), 100 µL phenol (1.5%), 50 µL peroxidase (15 U mL^{-1}) at 37°C for 30 min (Suzuki, 1981). The reaction was stopped by addition of (200 µL of 0.1 M potassium cyanide solution. In the blank, the solution of potassium cyanide was added to the mixture before the addition of the crude enzyme. The absorbance was measured against the blank in a spectrophotometer at 540 nm. One unit of uricase enzyme is defined as the amount of enzyme that produces 1 µmol of H_2O_2 per minute under the standard assay conditions.

Morphology and cultural characteristics: Spore chain morphology and the spore surface ornamentation of strain NEAE-25 were examined on starch nitrate agar medium after 14 days at 30°C. The gold-coated dehydrated specimen can be examined at different magnifications with Jeol JSM-6360 LA Analytical scanning electron microscope operating at 20 Kv at the Central Laboratory, City for Scientific Research and Technological Applications, Alexandria, Egypt. Color of aerial mycelium, pigmentation of substrate mycelium and diffusible pigments production were observed on ISP media 2-7 as described by Shirling and Gottlieb (1966) and on starch-ammonium sulphate agar; all plates were incubated at 30°C for 14 days.

Physiological characteristics: Physiological characteristics were performed following the methods of Shirling and Gottlieb (1966). The ability of the organism to inhibit the growth of four bacterial strains (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, or *Klebsiella pneumoniae*) and five fungal strains (*Rhizoctonia solani*, *Alternaria solani*, *Bipolaris oryzae*, *Fusarium oxysporum* and *Fusarium solani*) was determined. Some additional tests can be considered to be useful in completing the description of a strain or species, even if they are not very significant or indicative on their own, The ability of strain NEAE-25 to produce asparaginase (Gulati *et al*., 1997) and chitosanase activity (Choi *et al*., 2004) were tested.

Chemotaxonomy: The diagnostic whole-cell wall sugars were identified by the method described by Staneck and Roberts (1974).

16S rRNA sequencing: The preparation of genomic DNA of the strain was conducted in accordance with the methods described by Sambrook *et al*. (1989). The PCR amplification reaction was performed in a total volume of 100 µL which contained 1 µL DNA, 10 µL of 250 mM deoxyribonucleotide 5'-triphosphate (dNTP's); 10 µL PCR buffer, 3.5 µL 25 mM $MgCl_2$ and 0.5 µL Taq polymerase, 4 µL of 10 pmol (each) forward 16s rRNA primer 27f (5'-AGAGTTTGATCMTGCCTCAG-3') and reverse 16s rRNA primer 1492 r (5'-TACGGYTACCTTGTTACGACTT-3') and water was added up to 100 µL. The PCR-apparatus was programmed as follows: 5 min denaturation at 94°C, followed by 35 amplification cycles of 1 min at 94°C, 1 min of annealing at 55°C and 2 min of extension at 72°C, followed by a 10 min final extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The purified PCR product of approximately 1400 bp was sequenced by using two primers, 518F; 5'-CCA GCA GCC GCG GTA ATA CG-3' and 800R; 5'-TAC CAG GGT ATC TAA TCC-3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing product was resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) and deposited in the GenBank database under accession number HQ889312.

Sequence alignment and phylogenetic analysis: The partial 16S rRNA gene sequence of strain NEAE-25 was aligned with the corresponding 16S rRNA sequences of the type strains of representative members of the genus *Streptomyces* retrieved from the GenBank, EMBL, DDBJ and PDB databases by using BLAST program (www.ncbi.nlm.nih.gov/blast) (Altschul *et al*., 1997) and the software package MEGA4 version 2.1 (Tamura *et al*., 2007) was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was constructed via the neighbor-joining algorithm (Saitou and Nei, 1987) based on the 16S rRNA gene sequences of strain NEAE-25 and related organisms.

Fermentation conditions: Fifty milliliter of fermentation medium were dispensed in 250 mL Erlenmeyer conical flasks, sterilized and inoculated with previously prepared inoculum. The inoculated flasks were incubated on a rotatory incubator shaker at 200 rpm and 30-35°C. After the specified incubation time for each set of experimental trials, the mycelium of the isolate was collected by centrifugation at 6000 *g* for 15 min. The cell free supernatant was used as a crude enzyme for further determinations.

Statistical experimental design: Stepwise optimization strategy including, evaluation of the most significant medium constituents and environmental factors affecting enzyme production using Plackett-Burman factorial design (Plackett and Burman, 1946) and elucidation of the optimal concentrations of the most significant independent variables by a central composite design.

Selection of significant variables using Plackett-Burman design: The Plackett-Burman statistical experimental design is a two factorial design, very useful for screening the critical physico-chemical factors that influence the enzyme production

Table 1: Experimental independent variables at two levels used for the production of uricase by *Streptomyces rochei* strain NEAE-25 using Plackett-Burman design

		Levels	
Codes	Independent variables	-1	+1
X_1	Incubation time (days)	3.00	5.00
X_2	pH	6.00	8.00
X_3	Temperature (°C)	30.00	35.00
X_4	Inoculum size (%, v/v)	2.00	4.00
X_5	Inoculum age (h)	24.00	48.00
X_6	Medium volume (mL/250 mL flask)	25.00	50.00
X_7	Uric acid (g L^{-1})	2.00	5.00
X_8	Glycerol (g L^{-1})	10.00	30.00
X_9	KNO_3 (g L^{-1})	1.00	3.00
X_{10}	Yeast extract (g L^{-1})	0.00	1.00
X_{11}	K_2HPO_4 (g L^{-1})	1.00	2.00
X_{12}	$CaCl_2$ (g L^{-1})	0.10	0.30
X_{13}	NaCl (g L^{-1})	1.00	5.00
X_{14}	$MgSO_4.7H_2O$ (g L^{-1})	0.10	0.50
X_{15}	$FeSO_4.7H_2O$ (g L^{-1})	0.01	0.02

with respect to their main effects (Krishnan *et al*., 1998). Table 1 shows the independent variables under investigations, where different carbon sources (uric acid and glycerol), nitrogen sources (KNO_3, yeast extract), energy sources (K_2HPO_4), metals (NaCl, $CaCl_2$, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$) in addition to physical parameters like (incubation time, pH, temperature, inoculum size, inoculum age and medium volume) were tested. A total of 15 independent (assigned) and four unassigned variables (commonly referred as dummy variables) were screened in Plackett-Burman experimental design of 20 trials. Dummy variables (D_1, D_2, D_3 and, D_4) are used to estimate experimental errors in data analysis. Each variable is represented at two levels, high and low denoted by (+) and (−), respectively.

Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where, Y is the response or dependent variable (uricase activity); it will always be the variable we aim to predict, β_0 is the model intercept and β_i is the linear coefficient and X_i is the level of the independent variable; it is the variable that will help us explain uricase activity. All trials were performed in duplicate and the average of uricase activities were treated as responses.

Central composite design: The levels and the interaction effects between various variables which influence the uricase production significantly were analyzed and optimized using Central Composite Design (CCD). The highest three independent variables which obtained from Plackett-Burman experiment with respect to their main effect namely; incubation time (X_1), medium volume (X_6) and uric acid (X_7). In this study, the experimental plan consisted of 20 trials and the independent variables were studied at five different levels (-2, -1, 0, 1, 2). The central values (zero level) chosen for experimental design were: incubation time 5 days, medium volume 50 mL/250 mL flask and uric acid 6 g L^{-1}.

The experimental results of CCD were fitted via the response surface regression procedure, using the following second order polynomial equation:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j \quad (2)$$

In which Y is the uricase activity, β_0 is the regression coefficients, β_i is the linear coefficient, β_{ij} is the interaction coefficients, β_{ii} is the quadratic coefficients and X_i is the coded levels of independent variables. However, in this study, the independent variables were coded as X_1, X_6 and X_7. Thus, the second order polynomial equation can be presented as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_6 X_6 + \beta_7 X_7 + \beta_{16} X_1 X_6 + \beta_{17} X_1 X_7 + \beta_{67} X_6 X_7 + \beta_{11} X_1^2 + \beta_{66} X_6^2 + \beta_{77} X_7^2 \quad (3)$$

Statistical analysis: The experimental data obtained was subjected to multiple linear regressions using Microsoft Excel 2007. The p-values were used as a tool to check the significance of the interaction effects which in turn may indicate the patterns of the interactions among the variables (Montgomery, 1991). The statistical software package, STATISTICA software (Version 8.0, StatSoft Inc., Tulsa, USA) was used to plot the three-dimensional surface plots.

RESULTS

One hundred and thirty morphologically different actinomycetes strains were isolated and screened for their uricase activity using plate method (formation of clear zones around the colonies indicated the presence of uricase activity) (Fig. 1a). Out of these, 42% of the isolates exhibited urolytic activity during the preliminary screening experiment. Uricase producing isolates were categorized into 4 groups according to the width of inhibition zones; very strong (31-40 mm), strong (21-30 mm), moderate (11-20 mm) and weak (1-10 mm). The four groups were represented by 1, 16, 24 and 1% activity, respectively (Fig. 1b). Most promising isolate was selected and identified on the basis of morphological, cultural, physiological and chemotaxonomic properties, together with 16S rRNA sequence.

Morphology and cultural characteristics of the isolate NEAE-25: Morphology of strain NEAE-25 grown on yeast extract-malt extract agar (ISP medium 2) for 14 day revealed that strain NEAE-25 had the typical characteristics of the genus *Streptomyces*. Aerobic, gram-positive, mesophilic actinomycetes that develops abundant and well-developed substrate and aerial mycelium. It developed dark brown substrate mycelium, grey aerial mycelium on yeast extract-malt extract agar. Verticils are not present. The colour of the substrate mycelium was not sensitive to changes in pH. Strain

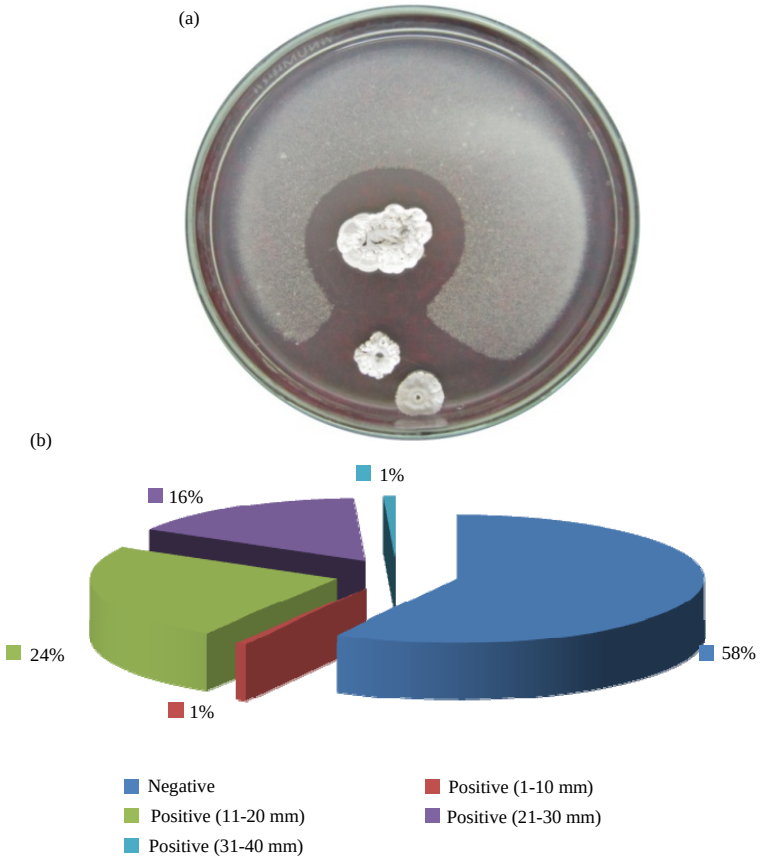

Fig. 1(a-b): (a) Screening of actinomycete isolates for uricase activity using plate method (formation of clear zones around the colonies indicated the presence of uricase activity), (b) Plate screening of actinomycetes isolates for the uricase activity (Percentage indicates the frequency of actinomycetes isolates within specific category in relation to the total isolates)

Table 2: Culture properties of the *Streptomyces* isolate NEAE-25

	Color			
Mediums	Aerial mycelium	Substrate mycelium	Diffusible pigment	Growth
ISP medium 2 (Yeast extract -malt extract agar)	Grey	Dark brown	Non-pigmented	Excellent
ISP medium 3 (Oatmeal agar)	Grey	Greyish brown	Non-pigmented	Good
ISP medium 4 (Inorganic salt-starch agar)	Grey	Faint grey	Non-pigmented	Excellent
ISP medium 5 (Glycerol asparagines agar)	Whitish grey	Grey	Non-pigmented	Very good
ISP medium 6 (Peptone-yeast extract iron agar)	Faint growth	Faint growth	Non-pigmented	Weak
ISP medium 7 (Tyrosine agar)	Grey	Dark brown	Non-pigmented	Excellent
Starch ammonium sulphate	Whitish grey	Faint grey	Non-pigmented	Very good

NEAE-25 grew well on ISP medium 2, 3, 4, 5, 7 and starch-ammonium sulphate agar medium. It exhibited poor growth on ISP medium 6. Diffusible pigments are not produced on any medium tested (Table 2). A scanning electron micrograph of spore chains of strain NEAE-25 cultured on starch nitrate agar medium revealed that the organism produced spirales spore-chains (Fig. 2). Spirals are closed or opened, sometimes almost flexuous. Spore chains are moderately long up to 50, or often more than 50 spores per chain. Spore surface is smooth.

Physiological and chemotaxonomic characteristics of the isolate NEAE-25: The physiological and biochemical reactions of strain NEAE-25 are shown in Table 3.

Melanoid pigments not formed in peptone-yeast extract iron agar or tyrosine agar. As the sole carbon source, it utilizes D-glucose, D-galactose, D-xylose, rhamnose, L-arabinose, D-fructose, cellulose and D-mannose for growth. Only trace of growth on sucrose and raffinose as the carbon sources. It degrades cellulose, casein, gelatin, starch and uric acid but not chitosan and L-asparagine. Nitrate reduction, gelatin liquefaction, starch hydrolysis (Fig. 3), milk coagulation and peptonization were positive, whereas lecithinase hydrolysis is negative. Growth occurs in the presence of NaCl up to 5% (w/v). Hydrogen sulphide production was negative. Strain NEAE-25 exhibited antifungal activity against *Alternaria solani* and *Bipolaris oryzae* but no antimicrobial activity

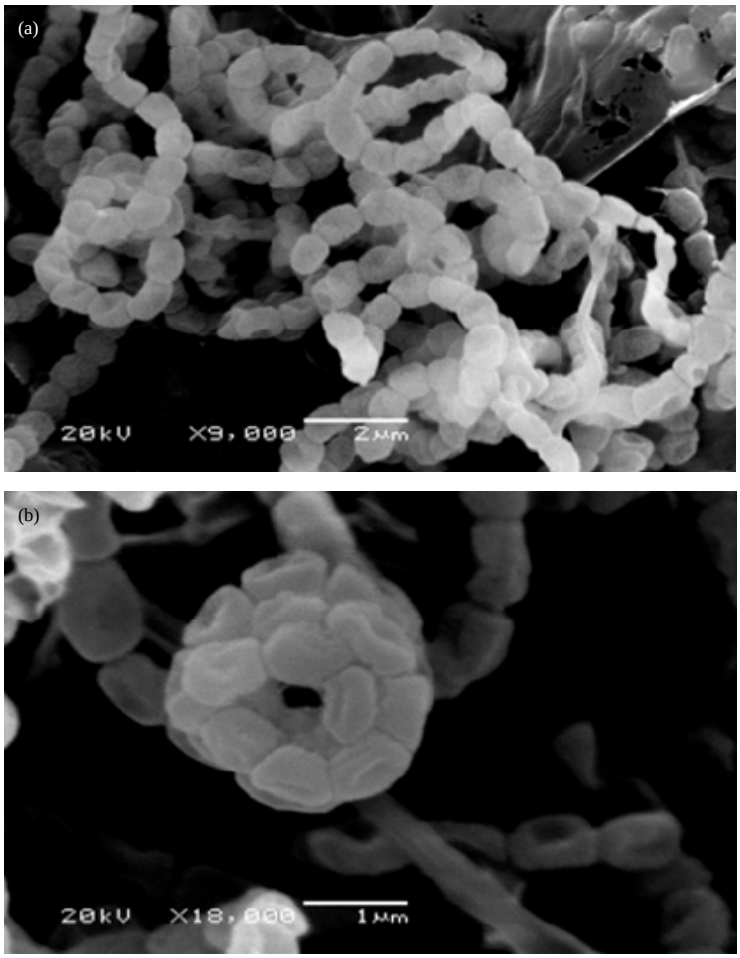

Fig. 2(a-b): Scanning electron micrographs showing the spore-chain morphology and spore-surface ornamentation of strain NEAE-25 grown on starch nitrate agar medium for 14 days at 30°C at magnification of, (a) 9000 X and (b) 18000 X. Spores in spiral chains and smooth surface of spores

against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella*, *Rhizoctonia solani*, *Fusarium oxysporum* or *Fusarium solani*. Chemotaxonomic tests showed that the whole-cell hydrolysates contained mainly mannose and arabinose.

Molecular phylogeny of the isolate NEAE-25: The almost-complete (1394 bp) 16S rRNA gene sequence of strain NEAE-25 was aligned with the sequences of the genus *Streptomyces* members retrieved from the GenBank databases by using BLAST (Altschul *et al*., 1997). The 16S rRNA gene sequence of strain NEAE-25 was deposited in the GenBank database under the accession number HQ889312. The sequence analysis revealed a close relationship to *Streptomyces rochei* strain AM 32 (GenBank accession no. JQ819732.1) with the maximum identity (99%). The phylogenetic tree (Fig. 4) showed that the isolate falls into one distinct clade with *Streptomyces rochei* strain AM 32 (GenBank accession no. JQ819732.1), *Streptomyces rochei*

Fig. 3: Plate assay showing zone of hydrolysis of starch by strain NEAE 25. All the starch in the medium near the microbe has been hydrolyzed by extracellular amylases

Table 3: Phenotypic properties that separate strain *Streptomyces* NEAE-25 from related *Streptomyces* species. Data for reference species were taken from Bergey's Manual® of Systematic Bacteriology-volume five, the actinobacteria (Goodfellow *et al*., 2012) and Wink (2012) electronic manual

Characteristics	*Streptomyces* sp. NEAE- 25	*Streptomyces rochei*	*Streptomyces djakartensis*	*Streptomyces albogriseolus*	*Streptomyces mutabilis*
Aerial mycelium on ISP medium 2	Grey	Grey	Grey	Grey	Grey or white color series
Substrate mycelium on ISP medium 2	Dark brown	Brown	Beige	Grayed yellowish brown or olive brown on yeast-malt agar	No distinctive pigment
Production of diffusible pigment	No distinctive pigment	No distinctive pigments		No pigments found in medium	Traces of yellow or greenish yellow pigments in yeast-malt agar
Spore chain morphology	Spirals are usually open, sometimes almost flexuous	Spirals are usually open, sometimes almost flexuous		Open spirals, flexuous or retinaculiaperti spore chains are also common	Spirales or retinaculiaperti
Number of spores /chain	With 10-50 or often more than 50 spores per chain	With 10-50 or often more than 50 spores per chain		10-50	3-10
Spore surface	Smooth	Smooth to warty		Smooth or warty	Smooth
Spore shape	Nearly spherical				Cylindrical
Sensitivity of diffusible pigment to pH	No pigments found in medium	No pigments found in medium		No pigments found in medium	Pigment is not pH sensitive
Melanin production on peptone-yeast extract iron agar (ISP medium 6)	-	-	+	-	-
Melanin production on tyrosine agar (ISP medium 7)	-	-	+	-	-
Max. NaCl tolerance (%, w/v)	5%	5%	5%	5%	
Degradation of					
Casein	+				
Gelatin	+	+	+	+	
Starch	+				
Utilization of carbon sources (1%, w/v)					
D (-) fructose	+	+	+	+	+
D (+) xylose	+	+	–	+	+
D (+) glucose	+	+	+	+	+
D (+) mannose	+	+	+	+	
Sucrose	±	±	+	+	±
D(+) galactose	+				
Cellulose	+		-	+	
Rhamnose	+	+	+	+	+
Raffinose	±	±	+	+	–
L-arabinose	+	+	+	+	+
Reduction of nitrates to nitrite	+				
Lecithinase activity	-				
H_2S production	-	-	-	-	
Coaggulation of milk	+				
Peptonization of milk	+				
Antimicrobial activities	+[1]	+	+	+[2]	

[1]Exhibited antifungal activity against *Alternaria solani* and *Bipolaris oryzae*. [2] Produce amphomycin and cephamycin antibiotics that active against gram positive bacteria. Produce vineomacin that has antibacterial and antitumor activities.[3]*Streptomyces* sp. NEAE- 25 in addition produces uricase and cellulase but not chitosanase and L-asparaginase and also utilizes maltose and ribose. RF: Rectiflexibiles, RA: Retinaculiaperti, S: Spirales, +: Positive, -: Negative, ±: Doubtful, Blank cells: No data available

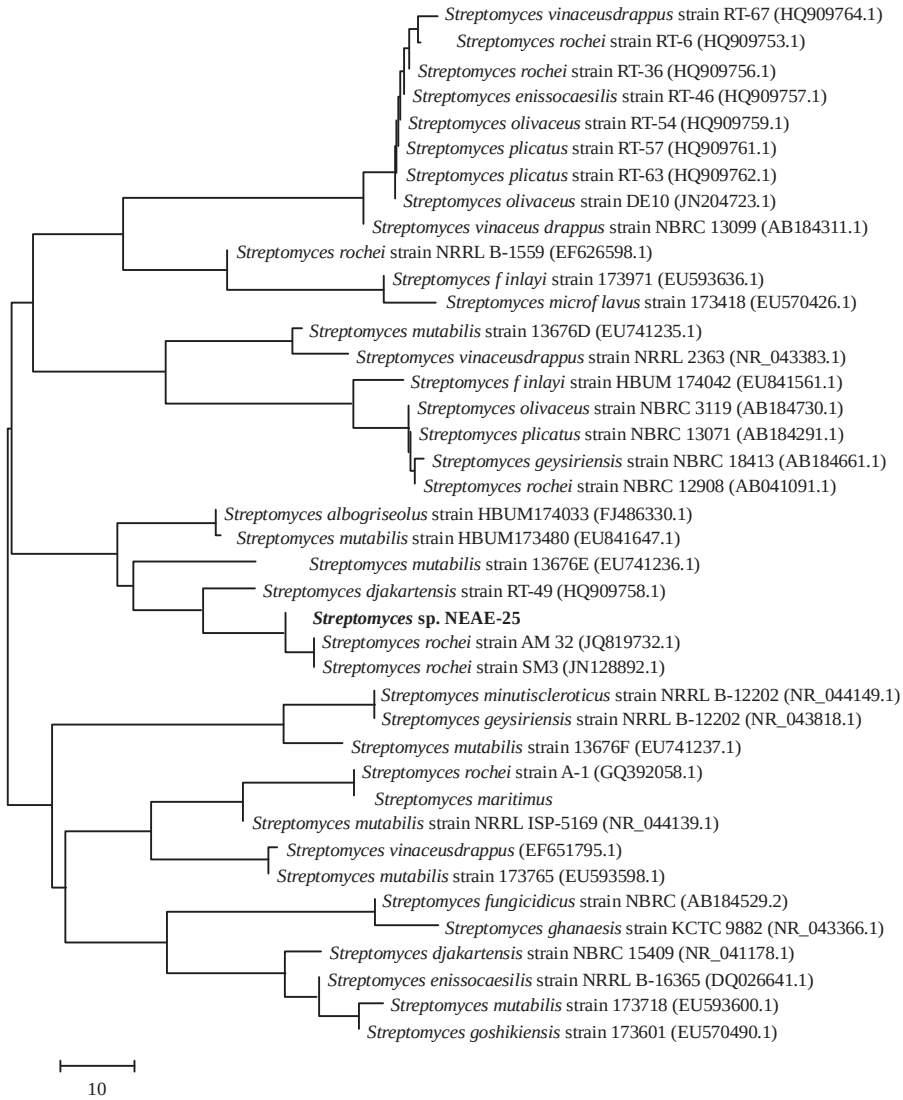

Fig. 4: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain NEAE-25 and related species of the genus *Streptomyces*. GenBank sequence accession numbers are indicated in parentheses after the strain names. Phylogenetic analyses were conducted in MEGA4

strain SM3 (GenBank accession no. JN128892.1), *Streptomyces djakartensis* strain RT-49 (GenBank accession no. HQ909758.1), *Streptomyces mutabilis* strain 13676E (GenBank accession no. EU741236.1), *Streptomyces mutabilis* strain HBUM173480 (GenBank accession no. EU841647.1), *Streptomyces albogriseolus* strain HBUM174033 (GenBank accession no. FJ486330.1) with which it shared 16S rRNA gene sequence similarity of 99.0%. It is clear that the strain NEAE-25 is closely similar to *Streptomyces rochei.*

Evaluation of the most significant factors affecting uricase activity: The experiment was conducted in 20 runs to study the effect of the selected variables on the production of uricase (Table 4). Fifteen different variables including medium component and physical parameters were chosen to perform Plackett-Burman experiment. The maximum uricase activity (43.58 U mL^{-1}) was achieved in the run number 11, while the minimum uricase activity (6.77 U mL^{-1}) was observed in the run number 18. The relationship between the independent variables and uricase production is determined by multiple-regression analysis. Statistical analysis of the uricase production was performed which is represented in Table 5. With respect to the main effect of each variables (Fig. 5), we can see that ten variables from the fifteen named incubation time, pH, temperature, inoculum size, inoculum age, medium volume, uric acid, yeast extract, $CaCl_2$ and $FeSO_4.7H_2O$ affect positively on uricase production, where the other five variables named (glycerol, KNO_3, K_2HPO_4, NaCl and $MgSO_4.7H_2O$) affect negatively on uricase production. The significant variables with positive effect were fixed at high level. The variables which exerted a negative effect on uricase production (glycerol, KNO_3, K_2HPO_4, NaCl and $MgSO_4.7H_2O$) were maintained at low level for further optimization by a central composite design. The model F value of 37.933 means that the

Table 4: Twenty-trial Plackett-Burman experimental design for evaluation of fifteen independent variables at two levels with coded values along with the observed uricase activity

Trials	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	D_1	D_2	D_3	D_4	Uricase activity (U mL^{-1})
1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	33.08
2	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	26.30
3	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	28.96
4	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	23.72
5	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	19.42
6	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	38.39
7	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	23.15
8	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	9.90
9	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	16.26
10	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	9.67
11	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	43.58
12	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	25.52
13	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	7.71
14	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	28.54
15	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	33.29
16	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	15.42
17	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	35.01
18	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	6.77
19	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	11.80
20	1	1	1	1	-1	1	-1	1	-1	-1	-1	1	1	1	-1	1	1	-1	-1	14.50

X_1-X_{15}: Represent independent (assigned) variables, D_1-D_4: Dummy variables (unassigned), 1: High level of variables and -1: Low level of variables

Table 5: Statistical analysis of Plackett-Burman design showing coefficient values, t-test, p-values and confidence level for each variable affecting uricase production by *Streptomyces rochei* NEAE-25

Variables	Coefficients	Main effect	t-stat	p-value	Confidence level (%)
Intercept	22.5471	45.0942	50.8676	0.00001	99.99
Incubation time (days)	5.8050	11.6090	13.0955	0.00020	99.98
pH	0.4250	0.8510	0.9594	0.39170	60.83
Temperature (°C)	1.6280	3.2570	3.6734	0.02130	97.87
Inoculum size (%, v/v)	0.6980	1.3970	1.5753	0.19030	80.97
Inoculum age (h)	2.2160	4.4320	4.9999	0.00750	99.25
Medium volume (mL/250 mL flask)	3.2480	6.4960	7.3271	0.00180	99.82
Uric acid (g L^{-1})	3.8350	7.6710	8.6530	0.00100	99.90
Glycerol (g L^{-1})	-2.4670	-4.9330	-5.5649	0.00510	99.49
KNO_3 (g L^{-1})	-0.0390	-0.0780	-0.0882	0.93390	6.61
Yeast extract (g L^{-1})	2.1050	4.2100	4.7484	0.00900	99.10
K_2HPO_4 (g L^{-1})	-0.4330	-0.8660	-0.9765	0.38410	61.59
$CaCl_2$ (g L^{-1})	3.0090	6.0180	6.7880	0.00250	99.75
NaCl (g L^{-1})	-4.7690	-9.5370	-10.7585	0.00040	99.96
$MgSO_4.7H_2O$ (g L^{-1})	-0.8190	-1.6380	-1.8475	0.13840	86.16
$FeSO_4.7H_2O$ (g L^{-1})	1.2010	2.4015	2.7090	0.05360	94.64

t: Student's test, p: Corresponding level of significance, Multiple R: 0.996503488, R Square: 0.993019202, Adjusted R Square: 0.96684121

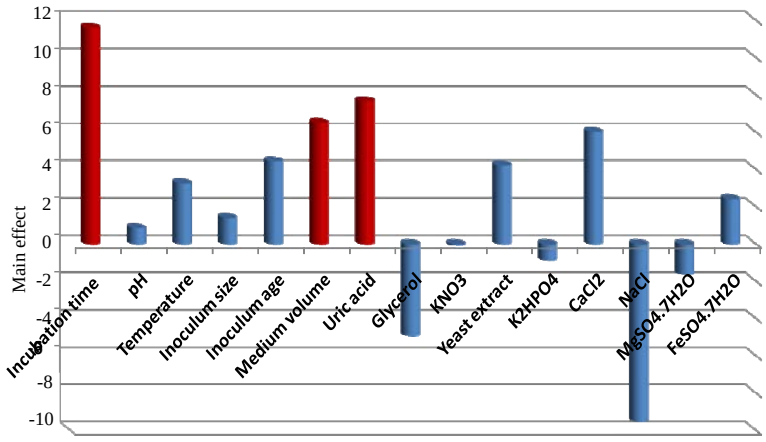

Fig. 5: The main effects of the fermentation medium constituents on uricase production by *Streptomyces rochei* NEAE-25 according to the Packett-Burman experimental results (The red color represent the most significant positive independent variables affecting enzyme production)

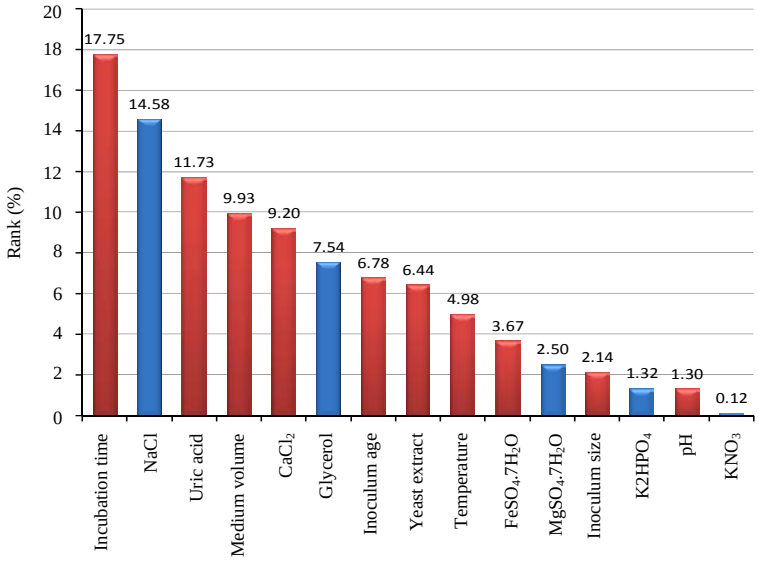

Fig. 6: Pareto chart illustrates the order of significance of the variables affecting uricase production by *Streptomyces rochei* NEAE-25 (the blue color represent negative effects and the red color represent positive effects; Ranks (%) values ranging from 0.12 to 17.75)

Table 6: Analysis of variance (ANOVA) for optimization of on uricase production by *Streptomyces rochei* NEAE-25 using Plackett-Burman design

	df	SS	MS	F	Significance F
Regression	15	2235.84133300	149.056088900	37.93336028	0.001507
Residual	4	15.71767835	3.929419588		
Total	19	2251.55901200			

df: Degree of freedom, SS: Sum of squares, MS: Mean sum of squares, F: Fishers's function significance, F: Corresponding level of significance

model is significant. The values of Significance F<0.05 (0.0015) indicate that model terms are significant Table 6.

The Pareto chart illustrates the order of significance of the variables affecting uricase production in Plackett-Burman experimental design (Fig. 6). Among the 15 variables, incubation time showed the highest positive effect by 17.75%, NaCl showed the highest negative significance by 14.58%.

In a confirmatory experiment, to evaluate the accuracy of Plackett-Burman, a medium of the following composition: (g L^{-1}: uric acid 5, glycerol 10, KNO_3 1, yeast extract 1, K_2HPO_4 1, $CaCl_2$ 0.3, NaCl 1, $MgSO_4.7H_2O$ 0.1 and $FeSO_4.7H_2O$ 0.02), incubation time 5 days, pH 8, temperature 35°C, inoculum size 4%, v/v, inoculum age 48 h and medium volume 50 mL/250 mL flask, gives 43.58 U mL^{-1} which is higher than the result obtained before applying Plackett-Burman (16.1 U mL^{-1}) by 2.7 times.

Optimization of medium composition using Central Composite Design (CCD): The CCD was employed to determine the optimal levels of the significant factors and also to study the interactions among these factors. The variables show highest main effects from Plackett-Burman experiment were selected to further investigation, the other variables in the study were maintained at a constant level which gave maximal yield in the Plackett-Burman experiments. The most significant positive independent variables affecting enzyme production named incubation time (X_1), medium volume (X_6) and uric acid (X_7) were further investigated using CCD. Each factor in the design was studied at five different levels (-2, -1, 0, 1, 2). The results of experiments are presented along with predicted response (Table 7). The results showed considerable

Table 7: Central composite design, with actual factor levels corresponding to coded factor levels, representing the response of uricase activity as influenced by incubation time, medium volume and uric acid

	Variables			Uricase activity (U mL^{-1})	
Trials	X_1	X_6	X_7	Measured	Predicted
1	-1(4)	1(60)	-1(5)	40.70	42.11
2	-1(4)	-1(40)	-1(5)	38.25	40.04
3	1(6)	-1(40)	1(7)	35.38	35.48
4	-1(4)	-1(40)	1(7)	38.78	38.91
5	0(5)	0(50)	-2(4)	42.48	40.43
6	0(5)	0(50)	0(6)	47.49	47.24
7	1(6)	-1(40)	-1(5)	33.67	34.69
8	0(5)	0(50)	0(6)	47.49	47.24
9	1(6)	1(60)	1(7)	40.18	39.91
10	-1(4)	1(60)	1(7)	38.55	39.04
11	-2(3)	0(50)	0(6)	39.78	38.62
12	0(5)	0(50)	0(6)	47.49	47.24
13	1(6)	1(60)	-1(5)	39.68	41.06
14	0(5)	0(50)	0(6)	47.49	47.24
15	0(5)	0(50)	0(6)	47.49	47.24
16	0(5)	2(70)	0(6)	36.71	35.96
17	0(5)	0(50)	2(8)	37.63	38.15
18	0(5)	0(50)	0(6)	47.49	47.24
19	2(7)	0(50)	0(6)	34.51	34.15
20	0(5)	-2(30)	0(6)	30.23	29.46

X_1: Incubation time, X_6: Medium volume, X_7: Uric acid

Table 8: Estimated regression coefficients, main effect, t-test, p-values and ANOVA for optimization of uricase production by *Streptomyces rochei* NEAE-25 using central composite design

Variables	Coefficients	Main effect	t -Stat	p-value
Intercept	47.2394	94.4787	93.7388	0.0000
X_1	-1.1194	-2.2388	-3.5439	0.0053
X_6	1.6243	3.2486	5.1424	0.0004
X_7	-0.5705	-1.1410	-1.8062	0.1010
X_1X_6	1.0744	2.1488	2.4052	0.0370
X_1X_7	0.4800	0.9600	1.0745	0.3078
X_6X_7	-0.4856	-0.9712	-1.0870	0.3025
X_1X_1	-2.7133	-5.4266	-10.7682	0.0000
X_6X_6	-3.6316	-7.2633	-14.4128	0.0000
X_7X_7	-1.9865	-3.9730	-7.8837	0.0000
Analysis of variance (ANOVA)				
	df	SS	MS	F	Significance F
Regression	9	531.1551	59.0172	36.9705	1.66E-06
Residual	10	15.9633	1.5963		
Total	19	547.1184			

t: Student's test, p: Corresponding level of significance, Multiple R: 0.98530, R Square: 0.97082, Adjusted R Square: 0.94456, df: Degree of freedom, SS: Sum of squares, MS: Mean sum of squares, F: Fishers's function, Significance F: Corresponding level of significance

variation in the uricase activity. Runs 6, 8, 12, 14, 15 and 18 showed a highest uricase activity (47.49 U mL^{-1}). The minimum uricase activity (30.23 U mL^{-1}) was observed in the run number 20. The uricase activity which is used was the average of a duplicate experiment made for all the cultures. The uricase activities in runs 6, 8, 12, 14, 15 and 18 were the mean of the six runs.

Multiple regression analysis and ANOVA: Multiple regression analysis was performed which is represented in Table 8.

Model verification: In order to determine the accuracy of the model and to verify the result, an experiment under the optimal conditions which obtained from CCD experiment was preformed and compared with the predicted data. The measured uricase activity obtained was 47.49 U mL^{-1}, closed to the predicted one 47.57 U mL^{-1} revealing that a high degree of accuracy.

The predicted optimal levels of the process variables for uricase production by *Streptomyces rochei* strain NEAE-25 were incubation time (5 days), medium volume (50 mL/250 mL flask) and uric acid (6 g L^{-1}).

DISCUSSION

On the basis of morphological, cultural properties above, together with the physiological and biochemical properties of strain NEAE-25 shown in Table 3, it is evident that strain NEAE-25 belongs to the genus *Streptomyces*. Strain NEAE-25 produced grey aerial mycelium and dark brown substrate mycelium on yeast extract-malt extract agar. The color of the substrate mycelium was not pH sensitive. The organism produced spirales spore chains. Spirals are closed or opened. Mature spore chains moderately long. Spore surface is smooth. Melanin or any diffusible pigments was not produced. A comparative study between strain NEAE-25 and its closest phylogenetic neighbours in morphological, cultural and physiological characteristics is summarized in Table 3. From the taxonomic features, the strain NEAE-25 matches with *Streptomyces rochei* in morphological, physiological and biochemical characters. Thus, it was given the suggested name *Streptomyces rochei* strain NEAE-25. The GenBank accession number of the sequence reported in this study is HQ889312.

Evaluation of the most significant factors affecting uricase activity: The R^2 value is always between 0 and 1. When R^2 is closer to the 1, this means that the model is strong and better to predict the response (Kaushik *et al*., 2006). In this study, the value of the determination coefficient (R^2 = 0.9930) indicates that 99.30% of the variability in the uricase production was attributed to the studied independent variables and only 0.7% of the variations are not explained by these variables. In addition, the value of the adjusted determination coefficient (Adj. R^2 = 0.9668) is also very high which indicates a high significance of the model (Akhnazarova and Kafarov, 1982). A higher value of the correlation coefficient (R = 0.9965) signifies an excellent correlation between the independent variables (Box *et al*., 1978), this indicated a good correlation between the experimental and predicted values.

Student's t-test and p-values determine the significance of each coefficient (Table 5). Larger t-value and smaller p-value imply the more significant corresponding coefficient (Akhnazarova and Kafarov, 1982). Some investigators have found that confidence levels greater than 70% are acceptable (Stowe and Mayer, 1966). Thus, in the current experiment, variables evidencing p-values of less than 0.06 (confidence levels exceeding 94%) were considered to have significant

effects on the uricase production. Incubation time, with a probability value of 0.0002, was determined to be the most significant factor, followed by NaCl (0.0004), uric acid (0.0010) and then medium volume (0.0018). Screened significant variables, incubation time (X_1), medium volume (X_6) and uric acid (X_7) exerted positive effects whereas, NaCl exerted a negative effect on uricase production. The model F value of 37.933 means that the model is significant. The values of significance F<0.05 (0.0015) indicate that model terms are significant (Table 6). On the basis of the calculated t-values (Table 5), incubation time (X_1), medium volume (X_6) and uric acid (X_7) were chosen for further optimization using central composite design, since these factors had the most positive effects on uricase production.

In this study, the best concentration of uric acid for maximum uricase production was 6 g L^{-1}, its concentration higher than 6 g L^{-1} did not enhance the enzyme productivity. Uric acid was used as inducer; high concentrations of uric acid probably inhibit the production of uricase. The optimal incubation time for attaining a higher uricase yield by *Streptomyces rochei* NEAE-25 was 5 days. Abdel-Fattah and Abo-Hamed (2002) reported that uricase produced from *A. flavus, Aspergillus terreus* after 4 days incubation and from *Trichoderma* sp. after 6 days while Kon *et al*. (1976) reported that maximum uricase produced by *Hyphomyces* after 5 days incubation. To study the effect of aeration on uricase production, different volumes of medium were tried in 250 mL flasks. A 50 mL production medium in 250 mL flask gave maximum uricase production. Dissolved Oxygen (DO) is important parameter in uricase production. Increased levels of dissolved oxygen have lead to enhanced uricase production.

By neglecting the insignificant variables (p>0.06), the first order polynomial equation was derived representing uricase production as a function of the independent variables:

$$Y_{(\text{uricase activity})} = 22.547+5.805\ (X_1)+1.628\ (X_3)+2.216(X_5) +3.248\ (X_6)+3.835\ (X_7)- 2.467\ (X_8)+2.105\ (X_{10})+ 3.009\ (X_{12})\ -4.769\ (X_{13})+1.201\ (X_{15}) \quad (4)$$

where, Y is the uricase activity and X_1, X_3, X_5, X_6, X_7, X_8, X_{10}, X_{12}, X_{13} and X_{15} are incubation time, temperature, inoculum age, medium volume, uric acid, glycerol, yeast extract, $CaCl_2$, NaCl and $FeSO_4.7H_2O$, respectively. It can be seen from equation 4 that glycerol and NaCl exerted negative effect on uricase production by *Streptomyces rochei* NEAE-25, while other factors exerted positive effect.

Optimization of medium composition using Central Composite Design (CCD): Multiple regression analysis was performed which is represented in Table 8. R^2-value of the regression model is higher than 0.9(0.9708) and this implies a very high correlation (Chen *et al*., 2009) and a very good fit between the experimental and predicted uricase production by *Streptomyces rochei* NEAE-25, the value of R is 0.9853. The value of the adjusted determination coefficient (Adj-R^2 = 0.9445) also confirms the significance of the model.

ANOVA of the regression model is required to test the significance and adequacy of the model (Table 8). It is evident from the very low probability value (1.66E-06) and Fisher's F-test (36.9705) that the model is highly significant. It can be seen from the t-values and p-values (degree of significance) listed in Table 8, that the linear coefficients of incubation time and quadratic effect of incubation time (X_1), medium volume (X_6) and uric acid (X_7) are significant, meaning that they can act as limiting factors in the production rate of uricase and little variation in their values will alter the production. On the other hand, the p-values of the coefficient suggest that among the three variables studied, incubation time (X_1) and medium volume (X_6) showed maximum interaction between the two variables (0.037), indicating that 97.3% of the model affected by these variables. Furthermore, among the different interactions, interaction between X_1, X_7, interaction between X_6, X_7 and linear coefficient of X_7 did not show significant effect on uricase production.

The second-order polynomial equation which defines uricase activity (Y) in terms of X_1, X_6 and X_7 (the independent variables) was obtained using multiple regression analysis:

$$Y_{(\text{uricase activity})} = 47.239-1.119X_1+1.624X_6 - 0.570X_7+1.074X_1 X_6+0.480X_1\ X_7\ -0.485X_6X_7-2.713X_1^2-3.631X_6^2-1.986X_7^2 \quad (5)$$

where, Y is the uricase activity, X_1, X_6 and X_7 are the incubation time, medium volume and concentration of uric acid, respectively.

Description of the interaction between bioprocess variables and the response are illustrated in three-dimensional plots (Fig. 7a-c) when one of the variables is fixed at optimum value and the other two are allowed to vary. Figure 7a shows that lower and higher levels of incubation time (X_1), medium volume (X_6) support relatively low levels of uricase production. On the other hand, the maximum uricase production clearly situated close to the central point of incubation time (X_1) and medium volume (X_6). Figure 7b shows that higher levels of the uricase production were attained with central point of incubation time (X_1) and uric acid (X_7). Further increase in the concentration of uric acid lead to the decrease in the uricase production. Figure 7c represents the interaction between medium volume (X_6) and uric acid (X_7). It showed that the maximum uricase production clearly situated close to the central point of medium volume (X_6) and uric acid (X_7).

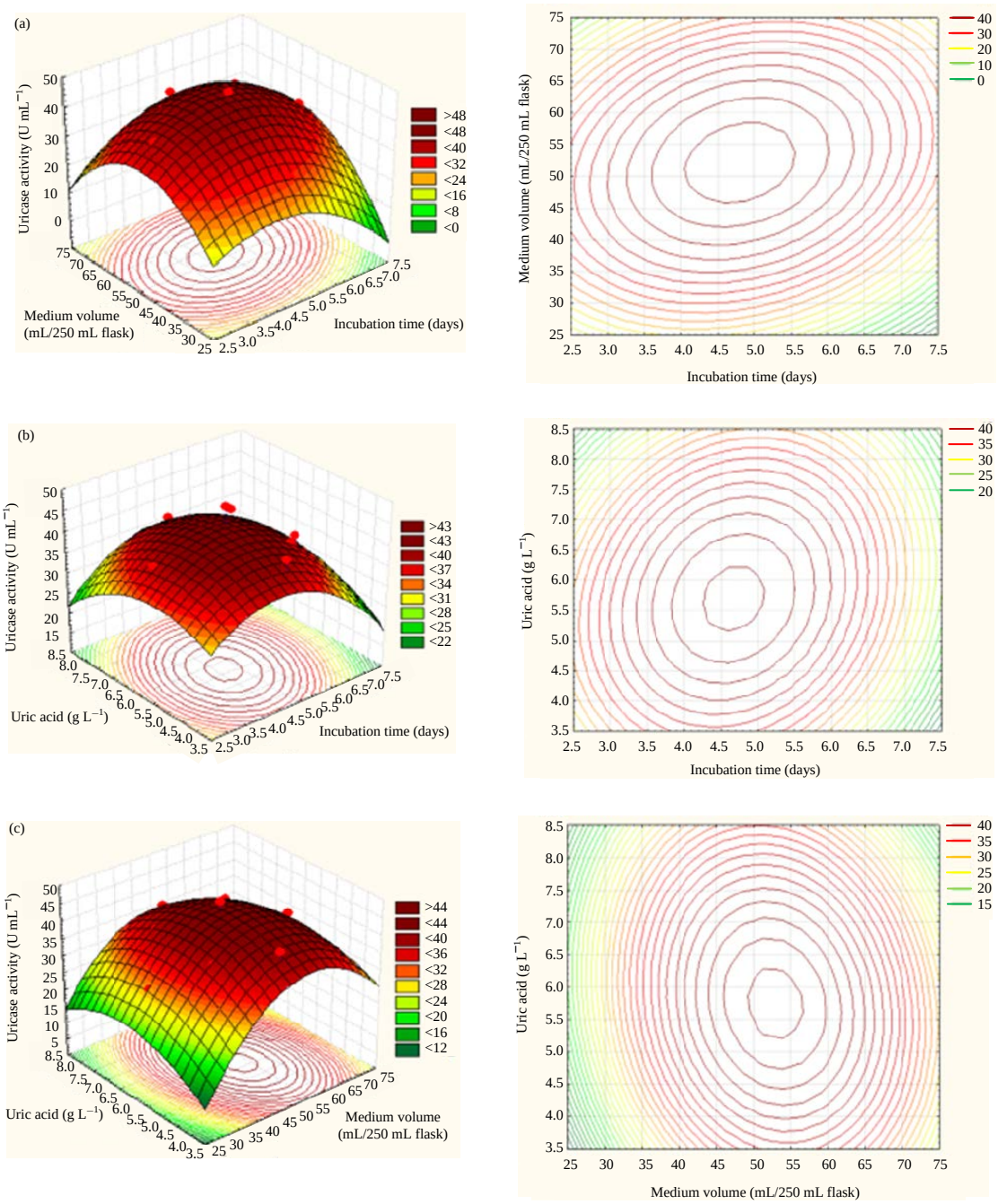

Fig. 7(a-c): Three-dimensional response surface and Contour plots showing the interactive effects of independent variables incubation time, medium volume and uric acid, on uricase production by *Streptomyces rochei* NEAE-25

CONCLUSION

The present study is to evaluate various parameters on uricase production by *Streptomyces rochei* NEAE-25 using response surface methodology and search optimal conditions to attain a higher uricase yield. As a result, a medium of the following formula is the optimum for producing an extracellular uricase in the culture filtrate of *Streptomyces rochei* NEAE-25: (g L^{-1}: uric acid 6, glycerol 10, KNO_3 1, yeast extract 1, K_2HPO_4 1, $CaCl_2$ 0.3, NaCl 1, $MgSO_4.7H_2O$ 0.1 and $FeSO_4.7H_2O$ 0.02), incubation time 5 days, pH 8, temperature 35°C, inoculum size 4%, v/v, inoculum age 48 h and medium volume 50 mL/250 mL flask. Significant improvement from 16.1-47.49 U mL^{-1} in the production of uricase.

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