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Statistical Optimization Of Media For The Production Of Uricase From Novel Actinomycetes *Streptomyces Ennisocaselis* A85

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Abstract

A powerful uricase producing actinobacteria was discovered as *Streptomyces ennisocaselis var* A85 after a comprehensive screening process. Utilizing response surface methodological optimization, the newly discovered *Streptomyces* strain produced more uricase. Plackett Burman (PB) design was utilized in media optimization studies to select the essential media components, which were then further optimized through the application of central composite design (CCD). Sucrose, uric acid and NaCl were discovered to be the key elements affecting the production of enzyme uricase. Through the use of central composite design, the best uricase production with these parameters was determined. Significant factors were of sucrose- 1.25g, peptone - 0.25g, NaCl - 0.03g, Na_2HPO_4 - 0.05g, $CaCl_2$ - 0.025g, uric acid - 0.107g, $MgSO_4.7H_2O$ - 0.025 for 50 mL of production medium. The utilization of statistical optimization resulted in a significant increase in uricase production, raising it from 3.33 to 17.00 U/mL. Overall, there was a 5.15-fold increase in uricase production accomplished by using the optimized medium with strain A85.

Keywords: Uricase, optimization, Plackett Burman design, central composite design, significant factors, anova

1. Introduction

Uric acid is a common component of urine. The IUPAC name of uric acid is 7,9-Dihydro -1H-purine-2,6,8(3H)-trione. The deposition of insoluble mono-sodium urate crystals in joints leads to severe, acute and chronic inflammatory arthritis, hyperuricemia and gout. *Streptomyces*, the most extensive type of Actinobacteria, serves as the representative genus for the *Streptomycetaceae* family [1]. Uricase (EC 1.7.3.3) is an oxidoreductase enzyme that is therapeutically significant. The nitrogen metabolism is highly reliant on the enzyme uricase. According to pitino *et al*., 2014 [2], uricase facilitates the process of oxidatively breaking the purine ring in uric acid to generate allantoin and hydrogen peroxide. Allantoin has greater solubility in comparison to uric acid. Due to several evolutionary processes, uricase is not functioning in humans, causing their serum levels of uric acid to be 3–10 times greater than those of other mammals [3]. Uricase was initially found to be valuable in clinical biochemistry as a diagnostic reagent for quantifying uric acid levels in blood and other biological fluids [4]. Uricase also can be used as a protein drug for the treatment of hyperuricemia as Rasburicase [5].

Uricase is endogenously synthesized by numerous plants, bacteria, and fungi, although microorganisms are the most dependable sources for large-scale industrial manufacturing. The uricase enzyme has been reported in many bacteria like *Bacillus subtilis* [6], *Pseudomonas aeruginosa* [7], *Escherichia marmote* [8] and fungal organsims like *Aspergillus welwitschiae* [9], *Gliomastix gueg* [10], *Gliocladium viride* [11]. The *Streptomyces* species which produce uricase are *Streptomyces rochei* [12], *Streptomyces exfoliate* [13], *Streptomyces albosrieolus* [14], *Streptomyces graminofaciens* [15], *Streptomyces aureofaciens* [16] *and Streptomyces cyanogens* [17].

When compared to other strategies for designing mediums, this method is straightforward and efficient for assessing the key characteristics that influence cultural needs [18,19]. Due to the multitude of components involved, certain factors may not have a substantial impact on the generation of bioactive metabolites. These designs are highly beneficial in initial investigations when the main goal is to identify factors that can be either changed or eliminated in subsequent optimization processes. Multiple researchers have utilized it to identify the influential factors among the components of a complex medium [20, 12]. A Plackett-Burman design is utilized to investigate the effects of eleven variables at both high and low levels.

The factors that have an impact can be improved by utilizing response surface approach, which can be implemented by either Central Composite Design (CCD) [21, 22] or Box-Behnken Design (BBD) experiments. Implementing these statistical experimental design techniques in medium optimization can lead to enhanced product yields, decreased process variability, reduced time, and total cost savings, when compared to the traditional approach of One factor at a time i.e. OFAT [23].

2. Materials and Methods

2.1. Strain used for production of uricase

Following the screening process, a strain with the ability to break down uric acid was identified and isolated. The isolate A85's genetic sequence (16S rRNA) was then uploaded to the Gene Bank (NCBI) with the Accession number: OR964862.1. Consequently, the strain A85 of *Streptomyces enissocaesilis* was identified as a new variant. This variant, derived from the isolate A85, was then utilized for the purpose of optimizing the production of uricase and enzyme activity was measured.

2.2. Uricase assay method

The assay for uricase activity was conducted by measuring the decrease in uric acid concentration at a wavelength of 293 nm using a UV-Spectrophotometer (Shimadzu UV 1800). The reaction mixture in the test contains 3mL of 20mM sodium borate buffer of pH 9.0, 50µL of 3.57 mM uric acid substrate solution and 50µL supernatant broth/ uricase enzyme. The blank contains 50µL buffer in place of uricase. The variation in absorption levels between the test and the blank is equivalent to reduction in uric acid concentration resulting from the enzymatic reaction [24]. One unit (u) of uricase activity was defined as amount of uricase is required to convert 1µmol of uric acid into allantoin per min at room temperature and pH 9.0, considering the millimolar extinction co-efficient of uric acid [20]. Uricase activity calculation formula can be given as:

$$\text{Units/mL enzyme} = \frac{(\Delta A_{293nm/min}\ \text{Test} - \Delta A_{293nm/min}\ \text{Blank})\ (B)\ (df)}{(12.6)(C)} \quad (1)$$

Where, ΔA = Absorbance
B = Total volume of reaction mixture
df = Dilution factor
C = Volume of enzyme
12.6 = Milli molar extinction coefficient of uric acid at 293 nm

2.3. Statistical experimental design for optimizing media

Optimizing media is crucial for enhancing enzyme synthesis. Initially, the effectiveness of different carbon and nitrogen sources was assessed. Traditionally used to increase the synthesis of enzymes. The statistical optimization experiments were conducted using STAT Ease software version 13.0, developed by Design Expert in Minneapolis, USA. The Plackett Burman design [25] was used to analyze the key factors that govern the synthesis of the uricase enzyme. The PB design revealed the most relevant elements, which were then further optimized via central composite design. The experimental sets were completed in triplicates, and the data was provided as the mean value with the standard deviation (SD).

2.4. Plackett Burman design for effective constituents

The current study [25] examined the 7 variables involving 8 runs (Table 1). The design matrix of PBD for conducting the experiments is displayed in (Table 1). Each column represents a distinct variable, while each row represents a trial, categorized as high (+1), center point (0), or low (-1), within each experimental trial (Table 2). The culture broth underwent fermentation in a 250 mL EM flask and was placed on a rotary shaker at a temperature of 28±2°C and agitated at a speed of 150 rpm for five days. The analysis was performed by the following equation:

$$E(xi) = 2\sum \frac{(Yi - Yi-)}{N} \quad (2)$$

Where $E(xi)$ is the concentration effect of the tested variable, $(Yi - Yi-)$ the uricase production from the trails where the variable (xi) was measured at high and low concentrations, respectively and N is the number of trails.

Table 1: Media composition in Plackett Burman design [25]

Std	Sucrose (g)	Peptone (g)	Uric acid (g)	Na_2HPO_4 (g)	NaCl (g)	$MgSO_4.7H_2O$ (g)	$Cacl_2$ (g)	Response (U/mL)
1	-1	-1	-1	1	1	1	-1	4.84 ± 0.1
2	1	-1	-1	-1	-1	1	1	9.91 ± 0.2
3	-1	1	-1	-1	1	-1	1	4.92 ± 0.1
4	1	1	-1	1	-1	-1	-1	10.0 ± 0.3
5	-1	-1	1	1	-1	-1	1	7.89 ± 0.2
6	1	-1	1	-1	1	-1	-1	9.99 ± 0.1
7	-1	1	1	-1	-1	1	-1	9.86 ± 0.3
8	1	1	1	1	1	1	1	10.0± 0.1

Table 2. Variables and levels of Plackett Burman design [25]

Media Components	Coded values	-1	0	+1
Sucrose	A	0.5	1.25	2.0
Peptone	B	0.1	0.25	0.5
Uric acid	C	0.07	0.107	0.185
Na_2HPO_4	D	0.03	0.05	0.1
NaCl	E	0.01	0.03	0.05
$MgSO_4.7H_2O$	F	0.01	0.025	0.05
$Cacl_2$	G	0.01	0.025	0.05

2.5. Central composite design for significant factors:

The Plackett Burman design [25] determined that sucrose (A), uric acid (B), and NaCl (C) were important components that were impacting the yield of uricase. The study examined these four factors across five distinct levels (-α, -1, 0, +1, +α) as outlined in Table 3. The model was fitted using a central composite design (CCD) that included 20 experimental runs with six replicates at the central points. Based on the experimental data using this methodology, a second-order polynomial regression model equation is derived as below:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C^2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 B \quad (3)$$

Here Y represents the projected reaction, while β_0 represents the intercept. Options A, B, and C are sucrose, uric acid and NaCl respectively. The response of uricase yield (Y) is determined by the coded independent variables (A, B, C) and the corresponding linear coefficients (β_1, β_2, β_3). The intercept term is represented by β_0. The quadratic coefficients are denoted as $\beta_1\beta_1$, $\beta_2\beta_2$ and $\beta_3\beta_3$. An analysis of variance (ANOVA) was employed to assess the experimental data. The optimal conditions for achieving the most uricase production were identified by solving the regression equation and analyzing the response surface, along with counter plots [20]

Table 3: Experimental variables and their levels used in CCD

Factors (%w/v)	Code	Range of levels				
		-α	**-1**	**0**	**+1**	**+α**
Sucrose (g)	A	0.011	0.5	1.25	2.0	2.51
Uric acid (g)	B	0.008	0.07	0.107	0.185	0.37
NaCl (g)	C	0.0036	0.01	0.03	0.05	0.06

3. Results and discussion

3.1. Screening of significant factors by placket Burmann design

The statistical analysis of the responses (Table 4) demonstrated that the model had a strong correlation with the test of significance, as indicated by the calculated p-value. The Model F-value of 89.00 and a p-value of 0.0500 suggest that the model has a high level of statistical significance. The PBD design yielded uricase activity ranging from 4.84 to 10.0 U/ml with uric acid, sucrose, and NaCl as significant factors. The probability of an F-value of this magnitude occurring solely due to noise is only 0.04%. The first order polynomial equation was generated to reflect uricase enzyme production as a function of the independent variable, by disregarding terms with a significance level greater than 0.072. The first-order polynomial equation (4) was obtained by regression analysis as follows:

$$R1 = 8.38 + 1.62A + 0.8750\,C - 0.8750E \quad (4)$$

The order of impact of the various variables was shown in a Pareto chart (Figure 1) using experimental data. The Pareto chart shows that Sucrose, NaCl, and Uric acid have the largest influence on uricase production by isolate A85. Hence these variables were further analyzed using response surface methodology.

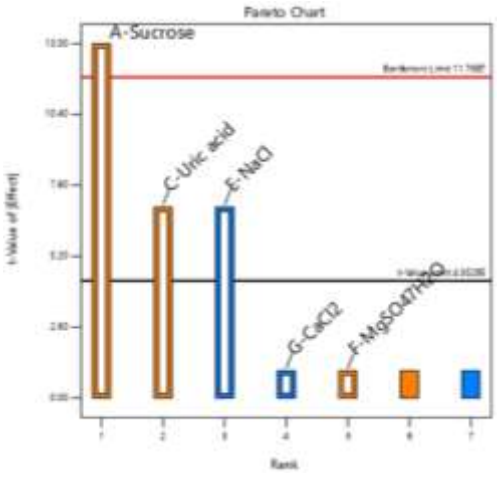


Fig.1. Pareto chart showing three critical components affecting uricase yield

Table 4: ANOVA for selected variables using Plackett-Burman design

Source	Sum of Square	df	Mean square	F-value	p-value
Model	33.38	3	11.13	89.00	0.0004
Sucrose	21.12	1	21.12	169.00	0.0002
Uric acid	6.12	1	6.12	49.00	0.0022
NaCl	6.12	1	6.12	49.00	0.0022
Residual	0.5000	4	0.1250		
Corrected total	33.88	7			

The significant model terms in this scenario are sucrose (A), uric acid (C), and NaCl (E). The R^2 values quantify the extent to which the experimental factors account for the variability observed in the experimental response values. The study R^2 determination coefficient value ($R^2 = 0.9852$) indicates that 98.52% of the variability in the answer can be attributable to the independent variables provided. The R^2 value should fall within the range of 0 to 1. A higher R^2 value indicates a stronger model and better predictive ability for the response variable [26]. Furthermore, the adjusted determination coefficient (Adj $R^2 = 0.9742$) has a remarkably high value, suggesting a strong level of relevance for the model. The coefficient of variation (CV) quantifies the extent of residual variation in the data in relation to the magnitude of the mean. Typically, tests with a greater CV value have lower dependability. A lower

coefficient of variation (CV) score of 4.22% suggests a higher level of reliability in the experimental performance. The model displays a standard deviation of 0.3536 and a mean value of 8.38.

The outcome exceeded the previously reported value by Pustake *et al.* [20] studied with three-level Plackett-Burman design [25] to evaluate the importance of 13 distinct characteristics in *Bacillus Subtilis* SP6. The presence of lactose, soya peptone, uric acid, and $FeSO_47H_2O$ were the key components that influenced the synthesis of uricolytic metabolites, resulting in activity levels ranging from 1.35 to 8.95 U/mL. In their study, El-Naggar [12] reported *Streptomyces rochei* NEAE-25 produced maximum 43.58 U/mL and minimum 6.77 U/mL in their PBD design and Incubation medium, volume and uric acid concentration are significant factors for production of uricase. In another study Sai Sushma and Vekateswarulu [8] performed experiments using *Pseudomonas mosselii* and determined that uric acid, sucrose, and peptone were important components. They observed uricase activity ranging from a minimum of 16.29 U/ml to a maximum of 51.25 U/ml after optimization. Similarly, Nevertheless, it was found that other factors were deemed insignificant, indicating that they did not impede the generation of uricolytic metabolites. Hence, the mutual reliance of significant variables cannot be sufficiently illustrated by employing a primary order equation. Consequently, the substances sucrose, sodium chloride, uric acid was selected for further used to determine the interaction effects of the independent input parameters through response surface methodology.

3.2. Medium optimization by CCD

Central composite design optimized important PB components. CCD experimental design used three components at two levels factorials. Table 3 shows high and low component levels and Table 5 shows the experimental design matrix and CCD analysis findings. The regression equation after ANOVA showed uricase production as a function of sucrose, uric acid, and NaCl. Based on quadratic regression analysis, CCD data showed the following second-order polynomial equation.

$$\text{Uricase activity} = 9.22 + 1.61A + 2.54B + 1.83C - 0.6363AB + 0.3263AC - 0.8663BC + 1.03A^2 - 0.8554B^2 + 0.8677C^2 \quad (5)$$

The suggested model has a model F value of 11.27, which is significant and has a 0.04% probability of being noise. Model terms (A, B, C, A^2, B^2, C^2) were significant when "prob F" was less than 0.05, whereas values over 0.1 indicated non-significance. A reasonable agreement between the anticipated $R^2 = 0.7429$ and the modified $R^2 = 0.8295$ indicated the model's ability to predict response. Precision, measured by signal-to-noise ratio, should be more than 4.0. The ratio is 11.7609. The model can travel design space. Table 6's "Lack of Fit F value" is 029, indicating that the result is not significant compared to the pure error. An excellent non-significant lack of fit indicated that the model equation may estimate uricase yield. The model's CV% 12.98 showed its accuracy and dependability.

Table 5: Experimental and predicted values of uricase yield recorded in the experimental setup of CCD

Std	Runs	Sucrose	Uricacid	NaCl	Uricase activity(U/mL)	
					Experimental	Predicted
1	4	0.5	0.07	0.01	4.98	4.80
2	16	2	0.07	0.01	9.00	8.64
3	18	0.5	0.3	0.01	14.09	12.90
4	17	2	0.3	0.01	14.29	14.19
5	19	0.5	0.07	0.05	9.85	9.55
6	2	2	0.07	0.05	13.90	14.70
7	5	0.5	0.3	0.05	14.22	14.18
8	9	2	0.3	0.05	17.01	16.78
9	3	0.011	0.185	0.03	8.59	9.41
10	20	2.511	0.185	0.03	15.09	14.83
11	8	1.25	0.008	0.03	7.53	7.36
12	10	1.25	0.378	0.03	15.18	15.91
13	11	1.25	0.185	0.003	7.69	8.59
14	6	1.25	0.185	0.063	15.09	14.76
15	1	1.25	0.185	0.03	8.50	9.22
16	15	1.25	0.185	0.03	8.50	9.22
17	14	1.25	0.185	0.03	8.50	9.22
18	7	1.25	0.185	0.03	12.90	9.22
19	13	1.25	0.185	0.03	8.50	9.22
20	12	1.25	0185	0.03	8.50	9.22

Table 6: Analysis of variance of main effects of factors for production of uricase

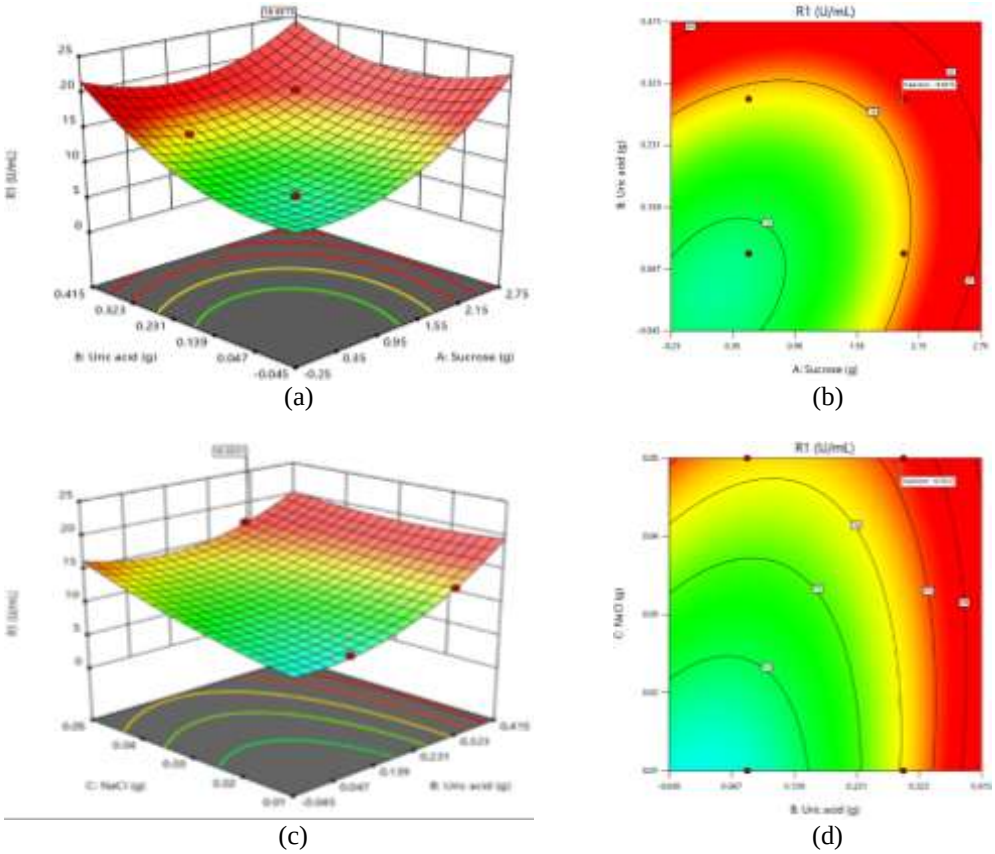
Factors	Sum of Squares	df	Mean sum of squares	F value	p-value Prob>F
Model	210.39	9	23.38	11.27	0.0004*
A-Sucrose	35.38	1	35.38	17.06	0.0020*
B-Uric acid	88.35	1	88.35	42.61	<0.0001*
C-NaCl	45.97	1	45.97	22.17	0.0008*
AB	3.24	1	3.24	1.56	0.2399
AC	0.8515	1	0.8515	0.4106	0.5361
BC	6.00	1	6.00	2.90	0.1197
A^2	15.20	1	15.20	7.33	0.0220*
B^2	10.54	1	10.54	5.08	0.0478*
C^2	10.85	1	10.85	5.23	0.0452*
Residual	20.74	10	2.07		
Lack of fit	4.60	5	0.9205	0.2853	0.9026
Pure error	16.13	5	3.23		
Corrected total	231.13	19			

* Significant values

Table 6 shows that sucrose and uric acid levels significantly affect uricase production, according to ANOVA. Sucrose and uric acid produced the most uricase under ideal conditions.

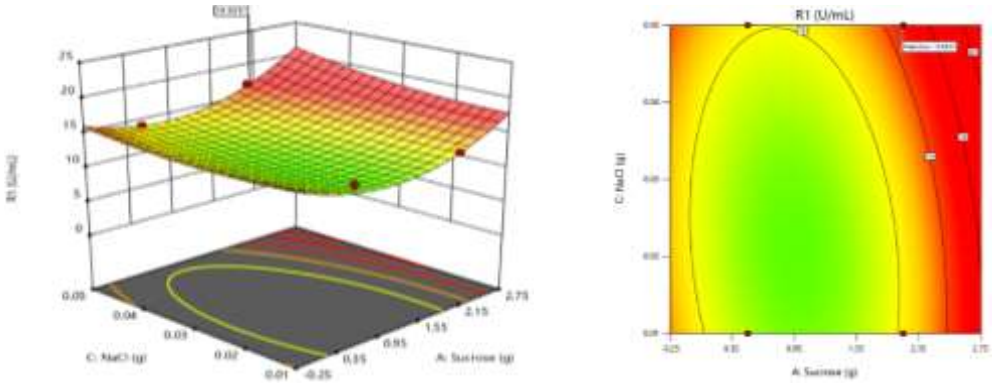
3.3. Interaction among the variables

Interactions between the significant variables for uricase enzyme production graphically studied by three-dimensional (3D) plots and two-dimensional (2D) contour plots [27]. Out of 3 variables, 2 kept at optimum level while one kept at zero level, to evaluate the yield of uricase enzyme. Three- dimensional (3D) plots and two-dimensional (2D) contour plots are straightforward and very comprehensible. The importance or insignificance of the 3D plot and 2D contour plots graphs are shown in Fig 2. is dependent upon the circular or elliptical nature of the contour plots. The circular order indicates interactions that are not significant, whereas the elliptical order indicates interactions that are substantial [21, 28]. Interaction between the sucrose and uric acid; uric and NaCl sucrose and NaCl; showed elliptical 2D contour plots, suggesting significant interaction in between them. Contour plots of these interactions are elliptical in nature, thereby implying significant interactions with each other.



(a) (b)

(c) (d)



(e) (f)

Fig. 2. Surface graphs for uricase production and interaction (a) 3D response of sucrose and uric acid (b) 2D response of sucrose and uric acid (c) 3D response of uric acid and NaCl (d) 2D response of and NaCl (e) 3D response of sucrose and NaCl (f) 2D response of sucrose and Nal

3.4. Validation of the model

A validation of model and regression equation is done by taking amount of sucrose - 1.25g, peptone - 0.25g, NaCl - 0.03g, Na_2HPO_4 - 0.05g, $CaCl_2$ - 0.025g, uric acid - 0.107g, $MgSO_4.7H_2O$ - 0.025, pH 9, distilled water 50 mL distilled water in the experiment. The maximum predicted yield of uricase 16.78 U/mL from A85 and the experimental response was 17.00 U/mL which shows that the model is validated.

Pustake *et al.* [20] conducted a study revealed that the concentrations for enhancing uricase enzyme production are as follows: 12.2g of lactose, 12.79g of soya peptone, 2.55g of uric acid, and 0.00325g of $FeSO_4.7H_2O$. The optimized medium exhibited significantly higher levels of uricase synthesis compared to the un-optimized media. The uricase production of *Bacillus subtilis* strain SP6 was measured to be 15.87 U/ml, whereas 7.1 U/ml for *Pseudomonas aeruginosa* [29], the predicted optimum uricase activity was which is 16.5 times than the basal medium. Another study *Streptomyces rochei* was measured to be 47.49 U/ml [12]. In this study, the best concentration of uric acid for maximum uricase production was 6g, its concentration higher than 6g did not enhance the enzyme productivity. Uric acid was used as inducer; high concentrations of uric acid probably inhibit the production of uricase.

Sai Sushma *et al.* [8] proposed optimum conditions of uric acid 4.67g, peptone 20.7g, and sucrose 28.7g were evaluated by conducting a fresh experiment and found that the uricase production from *Pseudomonas mosselii* was increased to 56.24 ± 0.12 U/ mL which is close to the predicted value of 55.78 U/mL.

4. Conclusion

This study presents the isolation of a highly efficient bacterium that produces uricase, as well as the optimization of uricase production. Recently the actinobacteria was identified as *Streptomyces ennisocaselis var* A85 SP6 by 16s rDNA sequencing. The essential parameters identified using PB design were sucrose, uric acid, NaCl. These factors were then adjusted using CCD. The utilization of a response surface methodology allowed for the optimization of a medium including basic carbon and nitrogen sources, resulting in a notable enhancement in the production of the uricase enzyme. The synthesis of uricase was increased by a factor of 5.15 in the optimized medium compared to the baseline production medium. It has the capacity to create 17.00 U/ml of uricase enzyme, which is more than any previously reported bacterium and comparable to many fungal uricase producers. The future scope of this research involves the chromatographic separation and purification of the uricase enzyme, as well as conducting a comprehensive biochemical and biophysical analysis of the enzyme.

Abbreviations:

NCBI : National Centre for Biotechnology Information.
mM : millimole
µmol : micromole
OFAT : One factor at -a- time
PBD : Plackett Burman Design
CCD : Central composite Design
RNA : Ribose Nucleic Acid

Conflicts of interest

The authors assert that they have no conflicting interests.

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