A PROJECT REPORT ON

Production of amylase from *Aureobasidium pullulans* and its optimization by Placket and Burman method.

Submitted to

Anekant Education Society's

Tuljaram Chaturchand College of Arts, Science and Commerce,

Baramati.(Autunomous) 413102 affiliated by Savitribai Phule Pune University, Pune.

As a partial fulfillment of the degree of

M. Sc. in Microbiology

Submitted by

Mr. Virkar Sumit Appa (15982).

Mr.Zagade Rohit Balu (15983).

Mr.Bhosale Shubham Ganesh (16033).

Mr.Sawant Akshay Sudam (16032).

Under the Guidance Of

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CERTIFICATE

This is to certify that, the work incorporated in the dissertation entitled **Production of amylase from *Aureobasidium pullulans* and its optimization by Placket and Burman method.** By Mr.Virkar Sumit Appa ,Mr. Zagade Rohit Balu, Mr. Bhosale Shubham Ganesh, Mr.Sawant Akshay Sudam were carried out under my supervision at the Department of Microbiology, as a partial fulfillment of M.Sc. degree in Microbiology under Tuljaram Chaturchand College, Baramati affiliated by Savitribai Phule Pune University, Pune. All the information in this dissertation is genuine to the best of my knowledge.

Research Guide

Department of Microbiology

Head

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DECLERATION

I hereby declared that this project report entitled "Amylase production from *Aureobasidium pullulans* by submerged fermentation and optimization of amylase production by Placket and Burman method "Written and submitted in supervision of Dr. Yogini R. Mulay. The imperial finding in this project on data collected by our self. The matter present in report is not copied I understand that any copied is liable to be punished in any way.

Mr.Virkar Sumit Appa.

Mr. Zagade Rohit Balu.

Mr. Bhosale Shubham Ganesh.

Mr.Sawant Akshay Sudam.

ABBREVATIONS

- YEME: Yeast extract malt extract.
- PDA: Potato dextrose agar.
- SDA: Sabouraud dextrose agar.
- AMFEP : Association of Manufactures of fermentation enzyme products
- GRAS: Generally regarded as safe.
- CCD: Central composite design.
- DNSA: 3,5-dinitrosalicyclic acid.

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ABSTRACT

Amylases are omnipresent which has a great demand in industrial market. In this study, the condition of amylase production from a fungal strain *Aureobasidium pullulan* were statistically optimized by applying Placket and Burman design under submerged fermentation conditions. Maximum enzyme activity (2.0×10^6 IU/ML) was founded in medium containing yeast extract 0.5gm, peptone 2.5gm, starch 5gm, MgSo4 0.01gm, KH2Po4 2.5gm, NaCl 0.75gm,CaCl2 0.0002gm,Distilled water 100 ml, PH 5.5. From pareto chart variables that were most significant for the amylase production were yeast extract & peptone.

Project Title

Production of amylase from *Aureobasidium pullulans* and its optimization by Placket and Burman method

Introduction and Review of Literature

Amylase was the first enzyme produced industrially from fungal source in 1894 which was used in pharmaceutical to treat the digestive disorder (Panday.A et al.2000). Many enzymes have been used till date for industrial purpose, which degrade starch, protein,cellulose, pectin etc . Enzymes have catalytic in nature, they can catalysed many reactions(Jha S.et al.2013).Starch is polysaccharide everywhere present in nature .It's stored the energy form in the plant. It can be degraded into oligosaccharides, disaccharides which is coverted into monosaccharides such as glucose by the action of enzyme called as Amylase(Yahya S.et al.2016).

Amylase has many application in fermentation, pharmaceutical, food, paper, sugar industries. Amylase accommodate about 25% of international market (Yahya S.et al.2016). Amylase is produced in the pancreas and salivary glands and is responsible for breaking down complex carbohydrates into simple sugars that can be absorbed by the body. Without amylase, the human body would have difficulty digesting starchy foods such as bread, potatoes, and rice. Elevated levels of amylase in the blood can indicate pancreatitis, a condition in which the pancreas becomes inflamed. Measuring amylase levels is therefore an important diagnostic tool in identifying and monitoring pancreatic disease. Amylase is a widely studied enzyme and is used in many biochemical and molecular biology research applications, including the study of enzyme kinetics and gene expression. Amylase has significant role in brewing and detergent industries. They are mostly used for starch liquefication to reduce their viscosity, production of maltose, oligosaccharides mixture and maltotetraose syrup. In detergent production they are used for increase the cleaning effect and used for the starch de-sizing in textile industry (R.vidyalakshmi et al.2009).

Amylase enzymes can be produced by a wide range of microorganisms, including bacteria, fungi, and yeast. Examples include *Bacillus subtilis, Bacillus licheniformis, Aspergillus oryzae,* and *Saccharomyces cerevisiae*. Amylase enzymes can also be obtained from non-microbial sources such as plants and animals. Examples of plant sources include sweet potatoes, barley, and wheat, while examples of animal sources include saliva and pancreatic tissue. Microbial amylase has a higher industrial demand. Amylase obtained from fungi have a significant role for industrial purpose. According to the Association of Manufactures of fermentation enzyme products (AMFEP), amylases for commercial needs are mostly produced by filamentous fungi.

Fungal amylase are mostly applicable for commercial production because fungal enzyme are more efficient than the bacterial enzyme (Yahya S.et al.2016).

Amylase is an enzyme that helps to break down complex carbohydrates such as starch and glycogen into smaller, more easily digestible sugars. There are several types of amylase enzymes, including alpha-amylase, beta-amylase, and gamma-amylase. The structure of amylase enzyme is primarily composed of amino acids, which are linked together to form long chains. The amino acid sequence of the enzyme determines its three-dimensional structure, which is critical to its function. The enzyme has a characteristic fold known as the (α/β)_8 barrel, which is formed by eight parallel β-strands connected by α-helices. The active site of amylase enzyme, where the carbohydrate substrates bind and are broken down, is located in a cleft in the center of the barrel. The active site contains several key amino acid residues that are essential for catalyzing the breakdown of carbohydrates. These residues include aspartic acid, glutamic acid, and histidine Overall, the structure of amylase enzyme is complex and crucial to its function in carbohydrate digestion. Understanding the structure of the enzyme can help researchers develop new therapies for diseases such as diabetes and obesity, which are associated with impaired carbohydrate metabolism.

Aureobasidium pullalans is ubiquitous, polymorphic and oligotrophic black yeast like a fungus. They observed mostly in tropical and temperate environment that occurs mostly in moist places (Singh R.et al.2015). *Aureobasidium pullalans* are conferred GRAS (generally regarded as safe) Status. It is has been used for industrial production of wide variety of enzymes used in biotechnology particularly in processes involving starch hydrolysis. The search for newer yeast is biotechnologically important for the development if efficient, economically safe amylolytic hydrolysis of starch (Mulay and Deopurkar, 2017).

The use of submerged fermentation is significant because of the ease of sterilization and process control is easier to engineer in this systems. Depending on the nature of strain and cultural condition, the enzyme can be constitutive or inducible, showing a various production patterned. The purpose of this work was to study the production of amylase by *Aureobasidium pollulans* in submerged fermentation and optimize the cultural condition for amylase production (R.vidyalakshmi et al.2009).

Plackett and Burman methods are experimental designs used in the optimization of industrial processes. These methods involve the use of a screening design to identify the significant factors affecting the process and then

use an optimization design to determine the optimal conditions for those factors. Analyze the results of the screening design to identify the significant factors affecting the production of amylase. Use statistical methods such as analysis of variance (ANOVA) to determine which factors have a significant effect. Validate the optimal conditions obtained from the optimization design by performing experiments under the optimal conditions and comparing the results with the predicted values. You can optimize the production of amylase using the Plackett and Burman method. It is important to note that this is a general outline and the specific details of the experimental design will depend on the particular system and process being studied.

The optimization by the Placket and Burman method, it is statistical software easy to handle with the help it can runs the many trail of experiment at a time. Placket and Burman design are successfully applicable to screen and optimize the process parameters in the bioprocess field.Central composite design (CCD) is mostly applicable for bioprocess optimization studies & it can gives the information about the interaction between variables, provide the information necessary for design and process optimization. The aim of the present work is to screen and optimize the process variables for amylase production from *Aureobasidium pullulans* using statistical techniques (Sethuraman Padmanaban et al. 2015).

Objectives

1. Isolation and Characterization of *Aureobasidium pullalan.*
2. Quantitative and Qualitative analysis of amylase produced by *Aureobasidium pullalans.*
3. Optimization of amylase production by Placket-Burman method.

Materials and methods

Collection of sample

The samples were collected from the walls of bathroom from locally area of Baramati, Pune.Moist blackening patches on the walls of bathroom were collected in zipper bag.

Isolation of *Aureobasidium pullulan*

Aureobasidium pullulan were isolated from walls of bathroom samples were inoculated into the following enrichment medium yeast extract malt extract (YEME) which contain yeast extract 0.3gm, malt extract 0.3gm, peptone 0.5gm dextrose 1gm distilled water 100 ml,PH 5-6. There was addition of chloramphenicol to avoid the bacterial contamination. It was kept on rotary shaker at 28°c at 120 rpm for 4 days. (Mulay and Deopurkar, 2017).

Samples were inoculated into the isolate medium potato dextrose agar (PDA) which contain Potato(peeled) 20gm, dextrose 2gm, distilled water 100 ml, PH 5-6. There was addition of chloramphenicol to avoid the bacterial contamination. It was kept on rotary shaker at 28°c at 120 rpm for 4 days. After incubation there was no sufficient growth observed.

Samples were inoculated into sabouraud dextros broth which contain Peptone 1gm,Dextrose 4gm,Distilled water 100 ml, PH 5.4. There was addition of chloramphenicol to avoid the bacterial contamination. It was kept on rotary shaker at 28°c at 120 rpm for 4 days. After incubation sufficient growth was observed (Mulay and Deopurkar, 2017). *Aureobasidium pullulan* was confirmed by performing the monochrome staining. 0.1ml sample from the flask was taken on the clean glass slide and heat fix it. After heat fixing there was addition of Bromocresol purple stain.

After the confirmation of yeast like cell of *Aureobasidium pullulan*, a loopfull liquid culture was streak on SDA plates which contain same contain, plates were incubated at 28°C for 48 hours.

Qualitative analysis

Qualitative screening was done by starch assay.

The amylase activity of *Aureobasidium pullulan* determined by starch assay.Sample was spot inoculated at centre on starch agar plate which contain starch 1gm, agar 3gm, distilled water 100 ml, PH7. Plates were incubated at 28°c for 4 days. After incubation plates were flooded with iodine solution(Miller, 1959).

Quantitative analysis

Inoculum Preparation

Aureobasidium pullulan inoculated into saburaud dextrose broth and incubated at 28°c for 48 hour on rotary shaker at 120 rpm. The cell count adjusted at 10^7cells/ml by haemocytometer (Mulay and Deopurkar, 2017).

Submerged Fermentation For Amylase Production

The Amylase production was done by submerged fermentation. The use of submerged fermentation is advantageous because in case of sterilization and process control. The production media for amylase production which contain 0.1% yeast extract, 1% starch, 0.5 % peptone,0.05 % MgSo4, 0.5% KH2PO4,0.15 % NaCl, & 0.0012 % CaCl2,PH 5.5. The production medium was inoculated with 10^7 cells/ml. The medium incubated at 28°c for 5 days on rotary shaker at 120 rpm (Mulay and Deopurkar, 2017).

Amylase Assay

Amylase assay was done by adding 0.1 ml enzyme (crude extract/fermented broth supernatant) into 0.9 ml 1% starch solution, incubated at 37°c for 20 min. After the incubation adding 1 ml DNSA (3,5-dinitrosalicyclic acid) & kept into boiling water bath for 10 min. Red colour was observed. After boiling add 9 ml distilled water & take OD at 540 nm. The experiment were performed twice (Kathiresan and Manivannan, 2006).

Optimization by Plackette and Burman method

The Placket and Burman design is used to screen the significant media components from large number of variables with minimum number of experiment. It is statistical software to run the multiple experiment at a time. The optimization was done by using Placket and Burman mini tab software. The twelve experimental runs were carried out to study the effect of seven medium components for amylase production. All the factors are prepared at two levels "+1" and "-1" and "+1" for high value and "-1" for low value (Yufeng Xie et al.2013).

Statistical design for optimization of amylase enzyme

Table 1: Factors of Placket and Burman design with their high and low levels (Yufeng Xie et al.2013).

Variables	Symbol	Factors levels (gm)	High level (gm)	Low level (gm)
Yeast extract	Y	0.1	0.5	0.02
Peptone	P	0.5	2.5	0.1
Starch	S	1.0	5.0	0.25
MgSo4	M	0.05	0.25	0.01
KH2PO4	K	0.5	2.5	0.1
NaCL	N	0.15	0.75	0.03
CaCL2	C	0.01	0.005	0.002

Table 2: The test results for seven factors of the applied design of Placket and Burman experimental design.

Trails	Y	P	S	M	K	N	C
1	+1	-1	-1	-1	-1	-1	-1
2	-1	+1	-1	-1	-1	+1	+1
3	-1	-1	+1	+1	+1	-1	+1
4	-1	-1	-1	+1	+1	+1	-1
5	-1	+1	+1	+1	-1	+1	+1
6	-1	+1	+1	-1	+1	-1	-1
7	-1	+1	-1	+1	-1	-1	-1
8	+1	-1	+1	+1	-1	+1	-1
9	+1	-1	+1	-1	-1	-1	+1
10	+1	+1	-1	+1	+1	-1	+1
11	+1	-1	-1	-1	+1	+1	+1
12	+1	+1	+1	-1	+1	+1	-1

(Y= Yeast extract, P = Peptone, S = Starch, M = MgSo4, K = KH2PO4, N= NaCl ,C= CaCl2).

Determination of Amylase activity

After the incubation of 5 days the fermented broth was centrifuged at 7000 rpm for 20 min at 4°c.The supernatant was taken as crude enzyme and determine it's Amylase activity. Take 0.1 ml crude enzyme and add 0.9 ml 1% starch, incubated at 37°c for 20 min, add 1 ml DNSA incubated at boiling waterbath for 10 min and add 9 ml distilled water . The OD was taken at 540 nm (Mulay and Deopurkar, 2017).

Protocol for standard graph of glucose

Stock solution (0.1M) prepared by adding 1.8gm of glucose in 100 ml of distilled water.

Table No 3: Protocol for standard graph of glucose.

Concentration of glucose (mM)	Amount of glucose (ml)	Distilled water (ml)	DNSA (ml)	Incubation period (min)	Distilled water (ml)	OD at 540 nm
10	0.1	0.9	1	10	8	0.46
20	0.2	0.8	1	10	8	0.71
30	0.3	0.7	1	10	8	0.79
40	0.4	0.6	1	10	8	0.90
50	0.5	0.5	1	10	8	0.94
60	0.6	0.4	1	10	8	1.12
70	0.7	0.3	1	10	8	1.30
80	0.8	0.2	1	10	8	1.32
90	0.9	0.1	1	10	8	1.35
100	1.0	0.0	1	10	8	1.39

Amylase Activity

The amylase activity was determined by the following formula.

$$\text{Amylase Activity} = \frac{\text{concentration of glucose from standard graph} \times 1000 \times 100}{\text{Volume of preparation} \times \text{incubation period.}}$$

One unit of enzyme activity is the amount of enzyme that releases 1 micromol glucose per minute and is expressed in U/10 Ml.

Result and Discussion:

Isolation: Sample collected from walls of bathroom inoculated in to YAME. After incubation of 4 days media turns in black colour.

Fig no1. Growth on broth after 4 days.

10 Sample from different walls of bath room were collected from different sources and processed for isolation of *Aureobasidium pullulan*

Fig no 2. Growth on plate after 48 hrs.

After incubation there was sufficient growth observed.

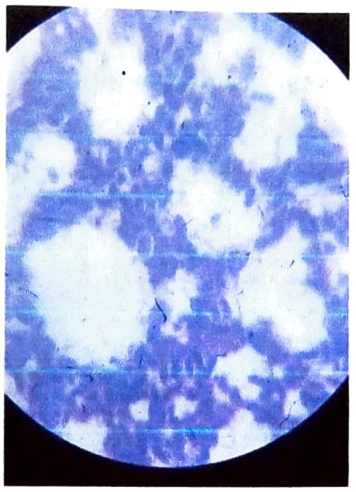
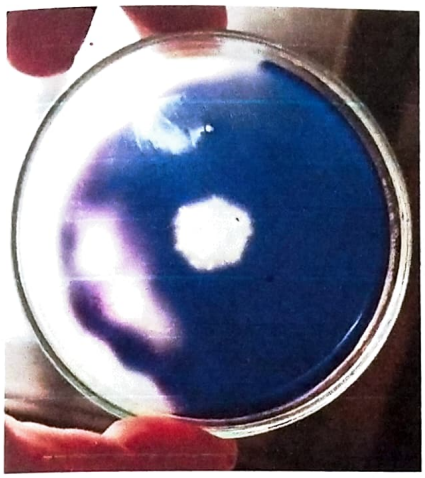


Fig no.2 Microscopic observation under 100 X.

. The yeast like fungal cells were observed under the oil emulsion lens (100X).microscope.

Amylase activity

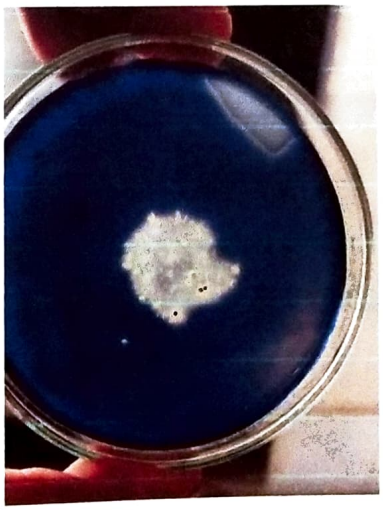
4 Different isolate of *Aureobasidium pullulan* were isolated and screen for amylase activity. All these isolate were tested for amylase production and result shown that strain no BB4 has shown maximum zone of clearance therefore this strain was selected for further studies.



Aureobasidium pullulan (BB1)

Aureobasidium pullulan (BB3)

Aureobasidium pullulan (BB2)



Aureobasidium pullulan (BB4)

Fig no.4 Starch degradation due to amylase production

The clear zone was observed around the spot of inoculation due to amylase production.

Inoculum Preparation

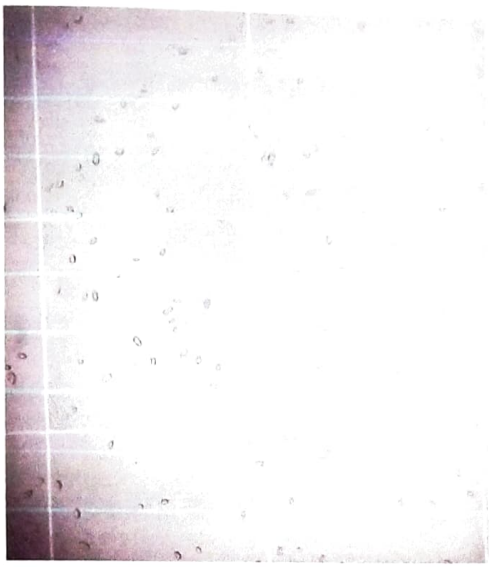


Fig no.5 Cells count under haemocytometer.

The yeast like cell was observed under haemocytometer and count it for preparation of inoculum for the production medium.

Amylase assay.

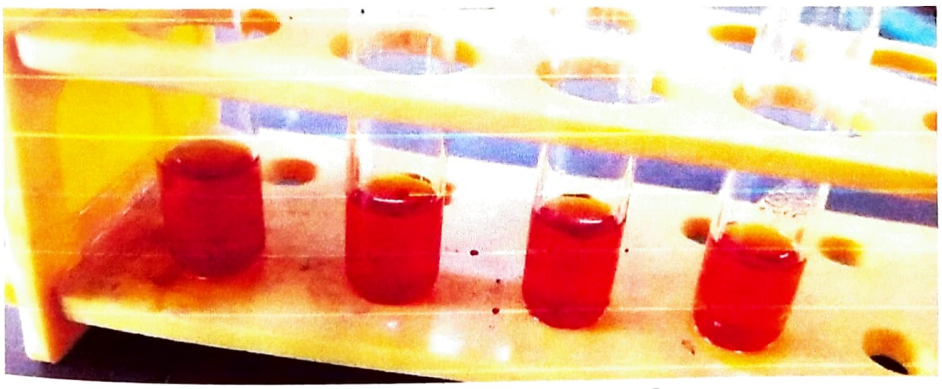


Fig no.5 Amylase assay of crude enzyme

Table no.4 OD of amylase assay.

Trials no	OD at 450 nm
1	0.065
2	0.045
3	0.078
4	0.057

Amylase assay of fermentation by Placket and Burman experimental design.

Fig no.6 Amylase assay of fermentation fermented broth.

Table No 5: OD at 540 nm of trails

Trails number	OD at 540 nm.
1	0.60
2	0.56
3	0.62
4	0.57
5	0.71
6	0.63
7	0.71
8	0.64
9	0.73
10	0.74
11	0.74
12	0.76

Standard graph of glucose

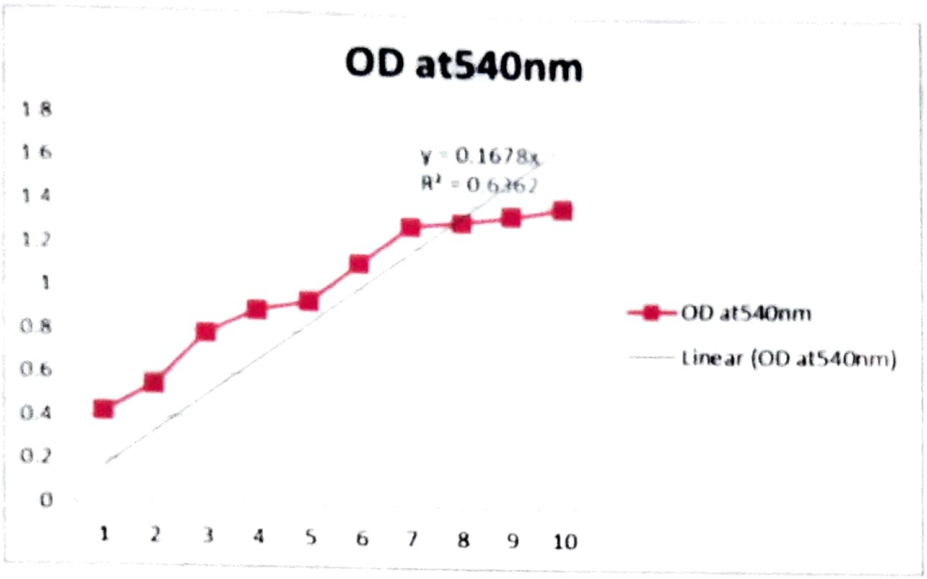


Fig no.6 standard graph of glucose

Amylase Activity

Table No 6: Twleve –trial Placket-Burman design matrix for seven variables with actual values along with observed yield of amylase.

S.O	R.O	Pt	Blocks	Y	P	S	M	K	N	C	Amylase activity (IU/10Ml)
12	1	1	1	-1	-1	-1	-1	-1	-1	-1	1.6×10^6
11	2	1	1	-1	1	-1	-1	-1	1	1	1.5×10^6
8	3	1	1	-1	-1	1	1	1	-1	1	1.6×10^6
9	4	1	1	-1	-1	-1	1	1	1	-1	1.5×10^6
7	5	1	1	-1	1	1	1	-1	1	1	1.8×10^6
3	6	1	1	-1	1	1	-1	1	-1	-1	1.7×10^6
2	7	1	1	1	1	-1	1	-1	-1	-1	1.8×10^6
4	8	1	1	1	-1	1	1	-1	1	-1	1.7×10^6
1	9	1	1	1	-1	1	-1	-1	-1	1	1.9×10^6
5	10	1	1	1	1	-1	1	1	-1	1	1.9×10^6
10	11	1	1	1	-1	-1	-1	1	1	1	1.9×10^6
6	12	1	1	1	1	1	-1	1	1	-1	2.0×10^6

the present study was conducted to statistically optimize the production and to partially characterize amylase from a fungal strain Aureobasidium pullalans (BB4).7factors were investigated to determine the optimum medium components suitable for amylase production. The amylase activities from the twelve runs are shown in Table 6. Fractional factorial Plackett Burman design was used to screen and evaluate the significant variables that can influence enzyme yield because this model does not explain the interaction among various variables (Motol & Agharkar, 1992). The results (Table 6) indicate a variation in amylase production in the range from 1.5 to 2.0 IU/10 ml by Aureobasidium pullalans (BB4). These variations revealed the importance of medium optimization to obtain better amylase yield (Kumar & Satyanarayan, 2007; Soni et al., 2006). Maximum Amylase activity was obtained in run number 12 containing (g/L); yeast extract (5.0), Peptone (2.5), starch (2.5), MgSo4 (0.01), KH2PO4 (2.5), NaCl (0.75), Cacl2 (0.03). The data on enzyme activity in Table 6 was subjected to multiple linear regression analysis to estimate t- and p-values of each component. On analysis of t-values, all factors have shown positive effect.

Placket-Burman Design

Factors: 7 Replicates: 1
Base runs: 12 Total runs: 12
Base blocks: 1 Total blocks: 1

Factorial Regression: C12 versus Y,P,S,M,K,N,C

Table No 7: Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	72.76667E+11	39523809524	2.40	0.208
Linear	7	72.76667E+11	39523809524	2.4	0.208
Y	1	2.13333+E11	2.13333E+11	12.96	0.023
P	1	20833333333	20833333333	1.27	0.323
S	1	20833333333	20833333333	1.27	0.323
M	1	833333333	833333333	0.05	0.833
K	1	13333333333	13333333333	0.81	0.419
N	1	0	0.00	1.00	0.537
C	1	7500000000	7500000000	0.46	0.537
Error	4	65833333333	16458333333		
Total	11	3.42500E+11			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
128290	80.78%	47.14%	0.00%

The statistical model itself is significant with a p-value of Yeast was 0.023 shows significant on amylase production(if P –value is lass than 0.05 shows significant effect and ,if P –value is greater than 0.05shows non significant on amylase production).

Coded Coefficients

Table No 8: Statistical analysis applied on Placket and Burman design for amylase production from ***Aureobasidium pullulan.***

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		1775000	3734	47.93	0.000	
Y	266667	133333	3734	3.60	0.023	1.00
P	83333	41667	3734	1.13	0.323	1.00
S	83333	41667	3734	1.13	0.323	1.00
M	-16667	-8333	3734	-0.23	0.833	1.00
K	666667	33333	3734	0.90	0.419	1.00
N	0	0	3734	0.00	1.000	1.00
C	50000	25000	3734	0.68	0.537	1.00

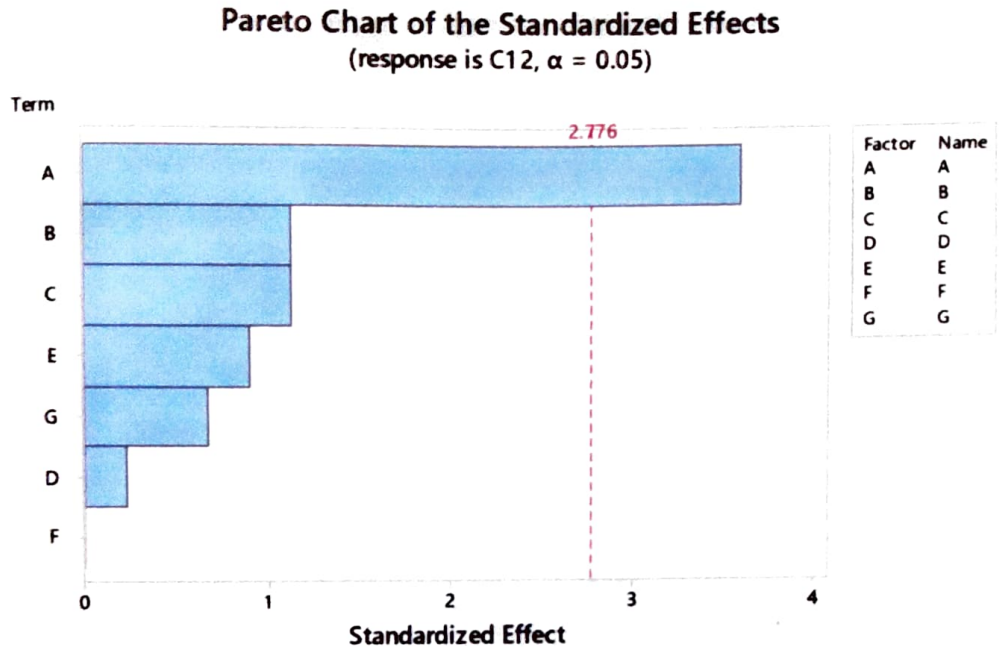
Regression Equation in Uncoded Units

C12 = 1775000 + 133333 A + 41667 B + 41667 C - 8333 D + 33333 E + 0 F + 25000 G

The data obtained from above table putted into multiple linear regression analysis to estimate t & p value of each content on the basis of t value all the factors show positive effect for amylase production except M which indicate negative effect(Saira Yahya et al.2016).

Pareto chart

Effects Pareto for C12



(A = Yeast extract , B = Peptone, C = Starch, E = MgSo4, G = KH2PO4, D = NaCl ,F = CaCl2).

Table Pareto chart for nutrient and conditions of significant variables for Amylase produce from ***Aureobasidium pullulans.***

Pareto chart was plotted for identifying significant factor for amylase production. It was evident from pareto chart the factor significant for amylase production by ***Aureobasidium pullulans*** were yeast extract (Saira Yahya et al.2016).

Conclusion

The result of present study indicate the possibility of Plackett-Burman trails for determining the factors that have positive effect on enzyme production .The amylase from Aureobasidium pullulan (BB4) .

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