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SCREENING OF NUTRITIONAL COMPONENTS FOR α-AMYLASE PRODUCTION IN SUBMERGED FERMENTATION BY BACTERIA ISOLATED FROM SOIL USING PLACKETT-BURMAN DESIGN

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ABSTRACT

Thermostable α-amylase can be produced by the bacteria isolated from soil in a liquid media. Screening of important nutrient parameters from selected seven medium components *viz.* starch, yeast, extract, tryptone, K_2HPO_4, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and KCl were carried out in shake flask cultures for enzyme production using Plackett-Burman experimental design of twelve trials. Among these starch, yeast extract and $CaCl_2.2H_2O$ contributes to large extent; tryptone and K_2HPO_4 have moderately significant; while $MgSO_4.7H_2O$ and KCl shows minute effect for amylase production by submerged fermentation.

Keywords: α-Amylase, submerged fermentation, Plackett-Burman design

INTRODUCTION

Microorganisms had significant contribution in production of various industrial enzymes. The global market for industrial enzymes estimated at $2 billion in 2004 and expected to rise at an average annual growth rate of 3.3%[1]. These starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile, paper, pharmaceutical to sugar industries[2,3,4]. Conversion of starch into sugar, syrups and dextrins forms the major part of starch processing industry[5]. Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits[6]. Nowadays, amylases (α-amylases, β-amylases and glucoamylases) represent one of the most important enzyme groups within the field of biotechnology[7]. α-amylase (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase, endoamylase) is a classical calcium-containing enzyme catalyze hydrolysis of starch and related carbohydrates by randomly cleaving internal α-D-(1-4) glycosidic linkage[8], yielding glucose, maltose, maltotriose, and other oligosaccharides[9]. It belongs to family thirteen in the classification of glycoside hydrolases. This family is the most varied of all glycoside hydrolase families, containing many enzymes able to catalyse various reactions, such as hydrolysis, transglycosylation, condensation and cyclization[10].

Amylase can be obtained from several fungi, yeast, bacteria and actinomycetes; however, enzyme from fungal and bacterial sources has dominated applications in industrial sectors[11]. The application of an amylase in industrial reactions depends on its unique characteristics, such as its action pattern, substrate specificity, major reaction products, optimal temperature, and optimal pH[12]. Bacterial α-amylase preferred for application in starch processing and textile industries due to its action at higher temperature (75-105^0C) and neutral to alkaline pH[13]. Generally production of this enzyme has been carried out by submerged fermentation[1,2]. Among the bacterial sources *Bacillus subtilis*, *B. staerothermophilus, B. amyloliquefaciens, B. licheniformis, B. acidocaldarius, Bifidobacterium* bifidum[1,14,15] and *B. macerans*[5] are important species. Amylase production could be induced by starch in a stable form. When cells

were grown on maltose glucoamylase production was much lower than on starch and amylase activity disappears after 24h growth on these media[16].

Designing a fermentation medium is a critical and vital process as the medium composition can significantly affect the product yield[17]. Screening of essential medium variables can be carried out by Plackett-Burman experimental design[18]. It is partial factorial design where large number of independent variables (N) are studied in small number of experiments (N+1)[19,20].

In the present study selected seven medium components were screened for their significance on amylase production by bacteria isolated from soil under submerged fermentation using Plackett-Burman design.

MATERIALS AND METHODS

Screening of microorganisms

Amylase producing bacteria were isolated from the soil collected from the garden of Amrutvahini College of Pharmacy, Sangamner, (Maharashtra, India) by streak plate technique using starch agar medium. It was maintained on nutrient agar salts throughout the experiment.

Plackett-Burman experimental design

The important nutritional parameters required for enzyme production in shake flask cultures using isolated bacteria were screened from selected seven medium components *viz*. starch, yeast extract, tryptone, K_2HPO_4, $CaCl_2.2H_2O$, $MgSO_4$, $7H_2O$ and KCl. The Plackett-Burman experimental design of twelve trials (Table 2) for eleven variables[18]: seven nutrient components (independent variables) and four dummy variables (Table 1) were used to evaluate the relative importance of various nutrients. In table 2, each row represents an experiment and each column represents different variable. For each nutrient variables two different concentrations high (+) and low (-) was tested (Table 1).

Table 1: Concentrations of various variables at different levels in Plackett-Burman design for amylase production in submerged culture.

S. No.	Designation	Variables	Low level(-) g/L	High level(+) g/L
1	X_1	Starch	5	15
2	X_2	Yeast extract	1	5
3	X_3	Tryptone	1	5
4	X_4	K_2HPO_4	3	7
5	X_5	$CaCl_2.2H_2O$	0	0.4
6	X_6	$MgSO_4.7H_2O$	0.1	0.9
7	X_7	KCl	0	0.4
8	X_8	Dummy1	-	-
9	X_9	Dummy2	-	-
10	X_{10}	Dummy3	-	-
11	X_{11}	Dummy4	-	-

Table 2: Plackett-Burman experimental design of 12 trials for eleven variables (+) high level, (-) low level along with observed enzyme activity

Trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Enzyme activity (mcg/ml)
1	+	+	-	+	+	+	-	-	-	+	-	0.423
2	-	+	+	-	+	+	+	-	-	-	+	0.245
3	+	-	+	+	-	+	+	+	-	-	-	0.284
4	-	+	-	+	+	-	+	+	+	-	-	0.261
5	-	-	+	-	+	+	-	+	+	+	-	0.164
6	-	-	-	+	-	+	+	-	+	+	+	0.125
7	+	-	-	-	+	-	+	+	-	+	+	0.437
8	+	+	-	-	-	+	-	+	+	-	+	0.409
9	+	+	+	-	-	-	+	-	+	+	-	0.384
10	-	+	+	+	-	-	-	+	-	+	+	0.157
11	+	-	+	+	+	-	-	-	+	-	+	0.336
12	-	-	-	-	-	-	-	-	-	-	-	0.144

Submerged fermentation

All experiments have been carried out in duplicates using 250 ml Erlenmeyer flasks containing 50 ml fermentation media, as per experimental design adjusted to pH 7.0 with 0.1M HCl or NaOH. These flasks were autoclaved at 15psi and 121°C for 15 minutes. Then each flask was inoculated with overnight culture of 1 ml cell suspension prepared in nutrient broth. Inoculated flasks were incubated on a rotary shaker incubator at 30°C and 150 rpm for 48 hours[1,21].

Enzyme extraction

Fermented medium were centrifuged to separate cells at 5°C and 5000g for 15 minutes. The clear supernants was used as crude enzyme extract[22].

Enzyme assay

The activity of amylase was estimated by incubating 0.3ml of crude enzyme extract with 0.5ml 1%w/v soluble starch prepared in 0.05M phosphate buffer of pH 6.5[16] for 10 min at 40°C[23,24]. The reaction was stopped by the addition of 1ml of 3,5-dinitrosalicylic acid reagent[25], the reaction mixture is heated in boiling water for 5 min. cooled and the reducing sugars released were analyzed by measuring the absorbance at 540nm[8]. An enzyme activity was expressed as the amount of reducing sugar released min^{-1} $milliliter^{-1}$.

Data analysis

1. The effect of each variable was determined with the following equation[27]:

   $E_{xi} = 2\ (\sum H_{xi} - \sum L_{xi}) / N$

   where, E_{xi} is the concentration effect of the tested variable, H_{xi} and L_{xi} are the concentrations of amylase at high level and low level of the same variable, and N is the number of trials (12). When the sign is positive, the influence of variable upon enzyme production is greater at high concentration, and when the negative, the influence of variable is greater at a low concentration.

2. Mean square of each variable (the variance of effect) were calculated as follows[27]:

   $V_{xi} = (\sum H_{xi} - \sum L_{xi})^2 / N$

where, V_{xi} is mean square of variable.

3. The experimental error was calculated by averaging the mean squares of the dummy variables[27]:

   $R = \sum V_{xd} / n$

   where, R is the experimental error (mean square for error), V_{xd} is mean square of dummy variable, and *n* is number of dummy variables (2).

4. Factor showing larger effects were identified using F-test[27]:

   $F = V_{xi} / R$

5. Percentage contribution was calculated from F-test.

RESULTS

Maximum enzyme production was found in experimental trial 7, where as, minimum in 12 (Table 2) under submerged fermentation using bacteria isolated from soil. Effect of all the four dummy variables close to zero (Table 3), indicates successful experimental work. Experimental error was calculated and was found to be 0.000.

Table 3: Influence of medium variables on amylase production

Designation	Variables	ΔH	ΔL	Mean Square	Effect	F value	% Contribution
X_1	Starch	2.273	1.096	0.115	0.196	979.89	78.06
X_2	Yeast extract	1.879	1.490	0.013	0.065	106.89	8.51
X_3	Tryptone	1.576	1.799	0.004	-0.038	37.02	2.95
X_4	K_2HPO_4	1.586	1783	0.003	-0.033	27.39	2.18
X_5	$CaCl_2.2H_2O$	1.866	1.503	0.011	0.061	93.28	7.43
X_6	$MgSO_4.7H_2O$	1.650	1.719	0.000	-0.012	3.39	0.27
X_7	KCl	1.736	1.633	0.001	0.017	7.47	0.60
X_8	Dummy1	1.712	1.657	0.000	0.009	-	-
X_9	Dummy2	1.679	1.690	0.000	-0.002	-	-
X_{10}	Dummy3	1.690	1.679	0.000	0.002	-	-
X_{11}	Dummy4	1.709	1.660	0.000	0.008	-	-

Among the seven selected parameters starch, yeast extract and $CaCl_2.2H_2O$ contributes to large extent, tryptone and K_2HPO_4 have moderately significant while $MgSO_4.7H_2O$ and KCl shows minute effect for amylase production in submerged fermentation by shake flask technique. Starch, yeast extract, $CaCl_2.2H_2O$ and KCl were influences the production at their higher concentration; whereas, tryptone, K_2HPO_4 and $MgSO_4.7H_2O$ were effective at lower level (fig.1).

DISCUSSION

Though amylases originate from different sources (plants, animals and microorganisms), the microbial amylases are the most produced and used in industry, due to their productivity, thermostability, suitability over wide pH range and biocompatibility[13]. Soil and food offer the substrates for isolation of microorganism strains producing amylases. In this respect, many strains used in food industry originate from fermented food media, while soils, particularly wastes and mud offer strains used mainly in chemical industry[27]. Production of amylase is highly dependent on starch[1,16] hence, it have maximum contribution. Starch is ubiquitous and is an easily accessible source of energy for bacterial growth. It is composed exclusively of α – glucopyranose units that are linked to

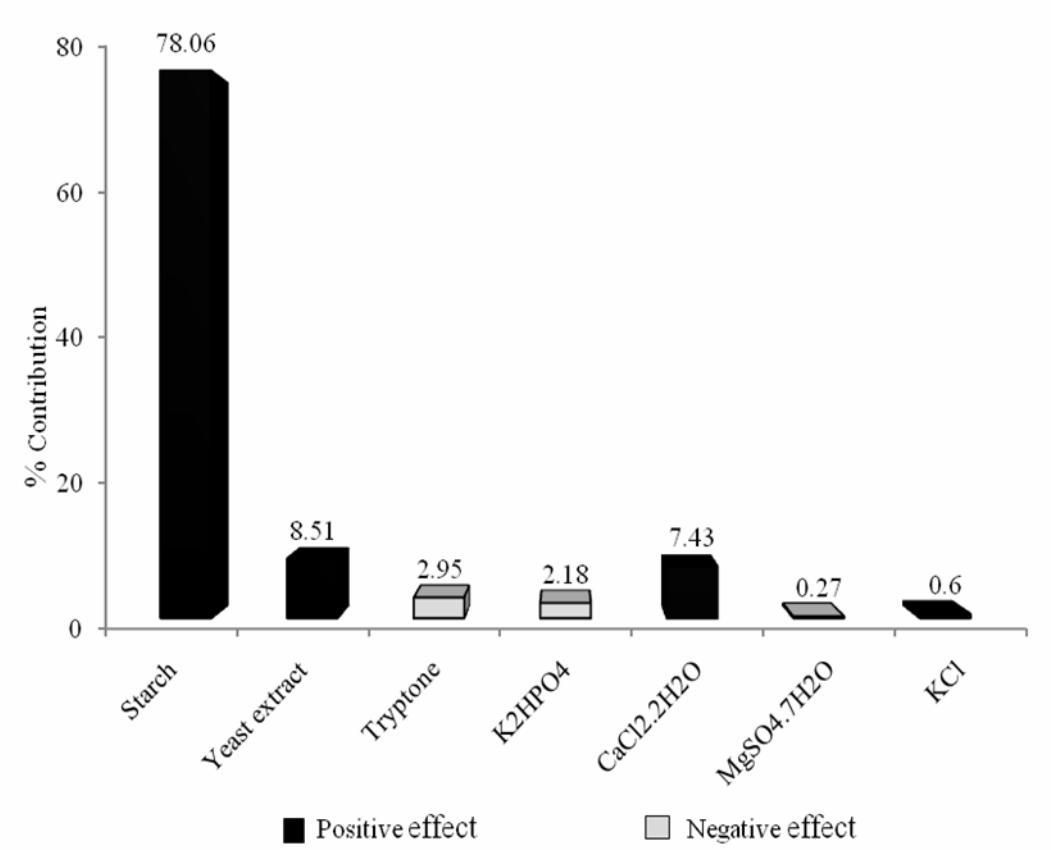

Fig. 1: Graph showing percentage contribution and relation of factors

other by α -1,4- or α -1,6-glucosidic bonds. The two high-molecular-weight components of starch are α amylose (representing a 15 to 25% weight fraction of starch), which is a linear polymer composed exclusively of α -1,4-linked glucopyranose residues, and amylopectin (representing a 75 to 85% weight fraction of starch), which is also an α -1,4-linked glucopyranose polymer but in addition contains α -1,6-glycosidic linkages representing branch points occurring at every 17 to 26 residues. Alpha amylase is capable of amylose degradation, yielding glucose, maltose, maltotriose, and other oligosaccharides by hydrolyzing α -1,4-glucosidic bonds[9]. These reducing sugars are utilized by the bacteria for further growth as carbon source. Yeast extract was found better nitrogen source than tryptone. Utilization of $CaCl_2.2H_2O$ at higher level indicates metal ions Ca^{2+} also helps in enzyme activity[8].

CONCLUSION

Designing the appropriate medium for the fermentation is an open ended, time consuming, laborious process involving large number of experiments. The Plackett-Burman experimental design is the preliminary technique for rapid illustration of the effects of various medium constituents. It tests each variable at two levels only; hence it can not give exact idea regarding the optimum level of constituent required in the medium. Therefore, further optimization of selected nutrients having greater contribution for amylase production is essential.

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