Statistical method based solid state fermentative bioprocess development for the production of polygalacturonase by *Rhizopus stolonifer*

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***Abstract*— Wheat bran is one of the major wastes from food processing industries, which poses considerable disposal problems and ultimately leads to environmental pollution. The main objective of the current research work was to determine the significant parameters on the production of industrially important polygalacturonase enzyme from wheat bran. Solid state culture conditions for polygalacturonase production by *Rhizopus stolonifer* from wheat bran were optimized by Taguchi's L-18 orthogonal array experimental design methodology. Eight fungal metabolic influencing variables, viz. temperature, moisture, wheat bran, inoculum, pectin, NH_4NO_3, $MgSO_4$, KH_2PO_4, and incubation time are used. The optimum conditions for predicted maximum polygalacturonase production were found to be pH 7, temperature 30 °C and fermentation period of 84 hrs.**

***Index Terms*— Polygalacturonase (PGase), solid state fermentation, Taguchi method, wheat bran.**

I. INTRODUCTION

Solid state fermentation is generally defined as the cultivation of microorganisms on solid materials in the absence or near absence of free water. In the recent years there has been a renewed interest in solid state fermentation (SSF) processes for production of bioactive compounds. SSF has been reported to be more advantageous than submerged fermentation (SMF) as it allows cheaper production of enzyme having better physicochemical properties than produced by SMF. Agricultural wastes and food processing wastes such as wheat bran, rice bran, sugarcane bagasse, citrus waste, and banana waste and fruit pomace are most commonly used substrates for SSF for industrially important pectinase production. Pectinases are used widely several industries. The physco-chemical parameters influence the microbial production of enzymes. Optimization of production conditions is very crucial for economic point of view.

Any fermentation process is significantly influenced by physical (pH, agitation and aeration) and chemical (medium constituents) parameters. In conventional one-parameter at a time methods, numerous experiments have to be carried out to optimize all the parameters (factors) and to establish best possible culture conditions by inter relating all the parameters. In recent years, use of statistical approach has gained lot of impetus for medium optimization for maximum production of enzyme and for understanding the interactions among various physicochemical parameters. Statistical experimental design techniques are very useful tools for this purpose, as they can provide statistical models that assist in understanding the interaction of different variables and predict the maximized product formation. The use of statistically designed experiments can allow the rapid and economical determination of the optimal culture conditions with fewer experiments and minimal resources. Alternative to one-parameter at a time method, researchers may want to test all combinations of parameters in an experiment, which is called a full factorial experiment. This strategy can cover all possibilities in the experiment and determine the optimal results. However, it will run too many trials, costing too much time and money in practice. Commonly used statistical approaches involved the use of Plackett–Burman (PB) [1]-[4] designs and Response Surface Methodology (RSM) [5], [6]. These methodologies have gained a lot of impetus for medium optimization and for understanding the interactions among various physicochemical parameters using a minimum number of experiments. In addition, these methodologies are suitable for describing a near optimum region and thus for exactly investigating conditions for a multifactorial system. Among the various computational designs, the Taguchi approach is widely used technique as the process reduces cost, improves quality, and provides robust design solutions. In order to solve the above difficulties, Taguchi's method was developed based on the concept of the orthogonal array (OA) [7], which can effectively reduce the number of tests required in a design procedure. It has been successfully applied in many fields such as chemical engineering, mechanical engineering, integrated chip manufacture, power electronics, and in bioprocess technology [8]-[13]. Taguchi considers three stages in product's or process's development: system design, parameter design, and tolerance design. In system design, the engineer uses scientific and engineering principals to determine the basic configuration. In parameter design stage, the specific values for the system parameters are determined. Finally, tolerance design is used to determine the best tolerances for parameters [14], 15]. In Taguchi's method, orthogonal arrays (OAs) are employed to optimize the amount of information obtained from a limited number of experiments.

The process of optimization through statistical design is a common practice in biotechnology. Medium optimization for fermentative processes is an important step for its commercial usage and involves a number of physicochemical parameters such as the composition of production medium, the carbon and nitrogen sources, minerals and trace metals, pH, temperature, aeration and inoculum size. Performance and feasibility of these processes depend significantly upon adopted horizon lengths, accuracy of each horizon, cost of

development, data period, frequency of revision, type of application, potential for automation, external and subjective data, pattern recognition capability, and the number of observations required. Sometimes improvement in the forecasting accuracy can yield considerable time and effort savings.

Enzymes involved in degradation of pectin are wide spread in nature and can be found in many plants, bacteria, and fungi. The most important enzymes of pectinase complex are polygalacturonase (EC 3.2.1.15), pectin lyase (EC 4.2.2.10), pectate lyase (EC 4.2.2.2), and pectin esterase (EC 3.1.1.11). Recently pectinases are more widely used in food, textile, fruit juice industry, and paper and pulp industries. Pectinolytic enzymes of fungal origin attract the most attention since they offer tremendous potential to the industry. In view of the potential applications of pectinases, production conditions were optimized for the maximum production of polygalacturonase using Taguchi method by newly isolated *Rhizopus stolonifer* VSL-8007 under SSF conditions and results were analyzed by qualiteck-4 software in this study.

II. MATERIALS AND METHODS

a. Materials

The media components namely pectin, ammonium nitrate (NH_4NO_3), magnesium sulphate ($MgSO_4$), potassium dihydrogen phosphate (KH_2PO_4) was obtained from Himedia Laboratories, India. Galacturonic acid was obtained from Sigma-Aldrich, Co., (USA).

b. Pectin peel powder

Pectin was obtained from orange peel collected from the local market. The peel was dried in hot air oven at 80 °C for 48 h, and extracted with hydrochloric acid and precipitated with alcohol dried and used as carbon source.

c. Microorganism and culture conditions

The fungal strain, *Rhizopus stolonifer*, was isolated from strawberry fruit and was identified at Agharkar Research Institute, Pune, India (Identification no. 8007). The culture is maintained on PDA agar slants at 4 °C. The spores were harvested from the 96-hours-old culture in 0.01% sodium chloride solution. The fungus was grown regularly in minimal mineral salt medium (MMS; K_2HPO_4-0.38 g/L, $MgSO_4$- 0.20 g/L, $FeCl_3$- 0.05 g/L, and NH_4NO_3- 1.0 g/L) supplemented with pectin (0.5%) at pH 5.5, 37 °C on orbital shaker rotating at 150 rev/min. The agar slants were sub-cultured at regular intervals. Spore suspension of 2×10^7 spores/g dry matter was prepared under sterilized conditions.

d. Fermentation conditions

For the production of polygalacturonase by *Rhizopus stolonifer* VSL-8007, MMS medium was prepared in 250 ml Erlenmeyer flasks and autoclaved at121 °C for 30 minutes. After cooling, the medium was inoculated with the 2×10^7 spores/g dry matter inoculum from the five day old slant culture at 37 °C temperature. The moisture content of the medium was adjusted approximately to 1:2.5 (weight of wheat bran in gm: moisture in ml). The fermentation was carried out for five days. After five days of fermentation, the contents of each flask were thoroughly mixed with 15 ml of sterile water and filtered through muslin cloth under vacuum. The filtrate was centrifused and supernatant was stored at 4 °C for further enzymatic assays.

e. Estimation of polygalacturonase (PGase) activity

The PGase assay was carried out according to the method described by Baldwin and Pressey [16]. The reducing sugars liberated were estimated using arsenomolybdate reagent according to Nelson and Somogyi [17]. One unit of PGase activity was defined as the amount of enzyme required to produce 1µmol of galacturonic acid per minute under standard assay conditions. The protein content of crude enzyme preparation was performed according to the method as described by Lowry et al [18].

f. Experimental design

The levels of the factors affecting the enzyme yield were optimized via the Taguchi method using the Qualiteck-4 software (USA). Eight fermentation factors, temperature, moisture, wheat bran, inoculum, pectin, NH_4NO_3, $MgSO_4$, KH_2PO_4, and incubation time were selected for the present Taguchi study. Three level factor variations were considered. The size of experimentation was represented by L-18 arrays. All the factors were assigned with three levels except temperature factor, which was assigned with two levels fermentation experiments were carried out using *Rhizopus stolonifer*, sub cultured and grown (96 h) in MMS medium. The diversity of factors was studied by crossing the orthogonal array (OA) of factors. Experimental results were fitted in Taguchi software to analyze further for individual and interactive influences, ANOVA and to know the contribution of each selected fermentation factor in the production of polygalacturonase enzyme by this fungal strain. Validation experiments were performed using optimized parameters of fermentation medium components and levels by software.

III. RESILTS AND DISSUCTION

Taguchi method was employed as a tool for systematic experimental design. It allows several effects of parameters to be simultaneously determined effectively and efficiently. The combination of standard experimental design techniques and analysis method in the Taguchi approach produces consistency and reproducibility rarely found in any other statistical method.

For determining optimal fermentation conditions above, optimal factor values were considered as level values while fixing the factor levels, and other factor levels, L1 and L3, were fixed as negative and positive respectively, considering the factor role in fungal metabolism and the fermentation experiments were executed. Table I indicates the selected fermentation factors and their levels for optimization of polygalacturonase production by *Rhizopus stolonifer*.

Factor	**Level 1**	**Level 2**	**Level 3**
Temperature (°C)	30	35	-----

Moisture (w/v)	1:2.0	1:2.5	1:3.0
Inoculum (%, ml, w/v)	5.0	10.0	15.0
Pectin (%,g, w/v)	0.50	1.0	1.50
NH_4NO_3 (%,g, w/v)	0.50	1.0	1.50
$MgSO_4$ (%,g, w/v)	0.10	0.20	0.30
KH_2PO_4 (%,g, w/v)	0.34	0.38	0.42
Incubation time (hrs)	60	72	84

Table I. Selected fermentation factors and different levels for polygalacturonase production by *Rhizopus stolonifer*-VSL-8007

The temperature factor was assigned only with two levels with nine level 1 and nine level 2 conditions (2^1), whereas the rest of the seven factors were assigned to three levels. Hence, these factors had six level 1, six level 2 and six level 3 conditions (3^7). Table II shows the experimental plan layout of the L-18 (2^1 X 3^7) orthogonal array used in this study.

Exp. No	Column								PGase production (U) Experimental value
	1	2	3	4	5	6	7	8	
1	1	1	1	1	1	1	1	1	5.714
2	1	1	2	2	2	2	2	2	8.163
3	1	1	3	3	3	3	3	3	2.448
4	1	2	1	1	2	2	3	3	2.857
5	1	2	2	2	3	3	1	1	1.632
6	1	2	3	3	1	1	2	2	4.081
7	1	3	1	2	1	3	2	3	16.0
8	1	3	2	3	2	1	3	1	11.020
9	1	3	3	1	3	2	1	2	2.040
10	2	1	1	3	3	2	2	1	0.000
11	2	1	2	1	1	3	3	2	0.775
12	2	1	3	2	2	1	1	1	2.448
13	2	2	1	2	3	1	3	2	1.224
14	2	2	2	3	1	2	1	3	1.632
15	2	2	3	1	2	3	2	1	0.000
16	2	3	1	3	2	3	1	2	1.632
17	2	3	2	1	3	1	2	3	1.224
18	2	3	3	2	1	2	3	1	0.816

Table II Fractional factorial design of L-18 (2^1 X 3^7) orthogonal array used for optimization of polygalacturonase production by *Rhizopus stolonifer*-VSL-8007

Variation of values in polygalacturonase production at assigned levels by this fungal strain was depicted in Table III. The difference between average value of each factor at higher level and lower level indicated the relative influence of the effect at their individual capacities. The positive or negative sign denoted variation of production values from level 1 to 2 or 3. Inoculum level, pectin and NH_4NO_3 showed positive impact with increase in their concentration, while moisture and KH_2PO_4 has negligible impact on polygalacturonase production, whereas temperature, $MgSO_4$ and incubation have negative influence. Maximum variation was observed with temperature and $MgSO_4$. Sub sector level data denoted that temperature and $MgSO_4$ factors caused negative influence on polygalacturonase production, while inoculum size, pectin and NH_4NO_3 showed positive effect with change in fermentation parameter values from level 1 to 2 (Table III). These data further confirmed that the physiological nutritional factors and their concentrations played a very important role in achieving a better enzyme production. Table IV indicates the interaction between two selected factors. The interaction was measured based on severity index value calculated by software program. These values between two selected factors varied with factors to factors. These results further confirmed that, each studied factor was important in enzyme production, and the influence of one factor on polygalacturonase production was dependent on the condition of the other factor in optimization of enzyme production by *Rhizopus stolonifer*, although they have different influence at their individual levels. The percentage contribution of each is shown in ANOVA (Table III). Incubation temperature was less significant when compared to other factors. The moisture level, inoculum size, pectin concentration, potassium dihydrogen phosphate (KH_2PO_4), and incubation time contributed equally for polygalacturonase production. The error observed was very low which indicated the accuracy of the experimentation.

Factors	Sum of squares	df	Mean square	F value	P value	R square	Percent
Temperature (°C)	26.11	2	13.05	4.776	0.0279	0.4235	16
Moisture (w/v)	27.22	2	13.61	1.993	0.1731	0.2216	17
Inoculum (%, ml, w/v)	25.53	2	12.76	2.253	0.1428	0.2435	17
Pectin (%,g, w/v)	27.13	2	13.56	1.904	0.1856	0.2138	17
KH_2PO_4 (%,g, w/v)	27.32	2	13.66	1.889	0.1879	0.2125	17
Incubation time (hrs)	26.13	2	13.07	5.272	0.0137	0.4296	17
Core total	159.44	12	-	-	-	-	~ 100

Table III. Analysis of variance (ANOVA) of experimental data on polygalacturonase production by *Rhizopus stolonifer* -VSL-8007

Factors	L1	L2	L3	L2- L1	L3- L1	L3- L2
Temperature (°C)	40.815	29.223	NA	-11.592	NA	NA
Moisture (w/v)	33.876	18.773	27.456	-15.103	-6.42	8.683
Inoculum (%, ml, w/v)	31.834	20.815	28.169	-11.019	-3.665	7.354
Pectin (%,g, w/v)	26.937	25.712	25.324	-1.225	-1.613	-0.388
KH_2PO_4 (%,g, w/v)	29.386	23.263	27.842	-6.123	-1.544	4.579
Incubation Time(hrs)	28.161	24.488	25.223	-3.673	-2.938	0.735

L1, level 1; L2, level 2; L3, level 3

Table IV Impact of a fermentation factors and their assigned levels on polygalacturonase production by *Rhizopus stolonifer*- VSL-8007.

The experimental data revealed that selected level 1 values of temperature, inoculum size and NH_4NO_3 of the medium was observed to be optimum for polygalacturonase production, where as pectin and KH_2PO_4, selected level 2 values, were noted to be good for optimal enzyme production. The other factors such as moisture influence, $MgSO_4$ and incubation time, level 3 concentrations were found to be better for polygalacturonase production, indicating the importance of physiological factors and media components along with their concentrations in polygalacturonase production by this fungal species (Table III). The optimized media composed of temperature (30°C), pectin (1.0 %,g, w/v), inoculum size (15.0%, ml, w/v), Moisture level (1:3.0 w/v), NH_4NO_3 (0.50 %,g, w/v), $MgSO_4$ (0.30%,g, w/v), KH_2PO_4 (0.38 %,g, w/v) and incubation time (84 hrs) for maximal production of extracellular polygalacturonase (16 units) by *Rhizopus stolonifer*.

IV. Discussion

Fungal polygalacturonase productions either alone or in combination with other pectinases like pectin methyl esterase and pectin lyase has several commercial applications in the food and beverage industries. In this study optimization of polygalacturonase production was carried out using wheat bran substrate by *Rhizopus stolonifer* under solid state fermentation process. Optimization of factors cannot be fully achieved until we know the interaction between the different factors to know best of its relevant microbiological and physiological properties of the desirable product are established within known limits of statistical validity. The environmental conditions may also affect the protein recovery and production of different pectinolytic enzymes in various organisms. Temperature, incubation time, pH and pectin as carbon source are the major environmental factors affecting polygalacturonase production by fungal species. These factors could affect the polygalacturonase production by *Rhizopus stolonifer* in solid state fermentation.

The previous methods of learning one variables at a time, while keeping all others at a predetermined level not only is very much inefficient but also very time consuming techniques and also ignoring the importance of interactions of various parameters at different concentrations and at different conditions. Taguchi approach of orthogonal array experimental design for media optimization, involving different sets of variables or factors over different levels and establish the relationship between variables and also the performance at the optimum level is obtained. Statistical optimization of polygalacturonase production by *Rhizopus stolonifer* was investigated under solid state fermentation process using L-18 orthogonal array of Taguchi methodology. This gives a clear view to understand the roles of different fermentation factors. We could characterize and design the experiment where the influence of the factors towards the process. The characters can be controlled such that a lower or a higher value in particular influencing factors produces the expected or preferred result. Thus, the levels of factors, to produce the best results can be predicted. Among the eight fungal metabolic influencing factors the pectin and inoculum size plays a more significant role than the other selected parameters.

The L-18 orthogonal array of Taguchi methodology, which helps in designing fermentation conditions for economic production. The imperative role of software mediated designs of experiments played a vital role in this respect with minimum experimentation and maximum production with in a stipulated time.

V. CONCLUSION

The present work showed that wheat bran an industrial waste could be a potential substrate for polygalacturonase enzyme production by newly isolated *Rhizopus stolonifer* under solid state fermentation. Optimization of the enzyme production by Taguchi's L-18 orthogonal array method is employed for the first time, which demonstrated clearly the impact of the process parameters on the yield of polygalacturonase. At the optimum conditions, the production of polygalacturonase was maximal. Thus the results obtained in this study could define that wheat bran waste from food processing industries as a suitable substrate for polygalacturonase production using solid state fermentation. These results lime light the industrial potential of wheat bran substrate as good raw material for polygalacturonase enzyme production and can prevent

environmental pollution and industrial waste management treatment problems.

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