Bulletin of Pure and Applied Sciences
Vol.39A (Zoology), No.1,
January-June 2020: P.53-61
Original Research Article

Print version ISSN 0970 0765
Online version ISSN 2320 3188
DOI 10.5958/2320-3188.2020.00007.8
Available online at www.bpasjournals.com

A Statistical Approach for Production of Pectinase through Optimization of Process Factors Using Mutant Strain of *Bacillus subtilis*

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Received on 27.01.2020
Accepted on 20.03.2020

Abstract:
The optimization of the various process parameters including nutritional and physiochemical factors was carried out for production of pectinase using from mutant strain of *Bacillus subtilis* using Taguchi methodology. In the entire optimization study, mutant UV-20-11/NTG-1-5/EMS-1-2 was used. The experimental work was performed using shake flask experiments. In this study, ten factors including, orange peel, ammonium sulfate, magnesium sulfate, phosphate ion, sodium chloride, inoculum percentage, temperature, pH, agitation speed, and incubation time were considered for their effect on pectinase production. It was evident from Taguchi methodology that agitation speed contributed the maximum impact (26.51%) on pectinase productivity followed by ammonium sulfate (13.77%), incubation time (12.77%), temperature (12.36%), pH (V%), orange peel (9.64%), inoculum percentage (5.61%), and sodium chloride (5.467%). The results showed that a higher level of pectinase productivity (630.58 U) was achieved with orange peel (5 g/L), ammonium sulfate (1.5 g/L), magnesium sulfate (0.5 g/L), phosphate ion (0.5 g/L), sodium chloride (0.5 g/L), inoculum percentage (1%), temperature (37^{o}C), pH (7), agitation speed (140 rpm), and incubation time (96 h).

Keywords: Taguchi methodology, optimization, pectinase**,** mutant, process factors

INTRODUCTION

Enzymes are biochemically protein in nature. They are synthesized by living system. They performed the catalyses (Bhunia et al., 2012; Bhunia et al., 2013)and therefore, are very important in synthetic (anabolism) as well as derivative processes (catabolism).Industrial important enzymes are critical components of numerous industries such as bio-pharmaceutical, food, paper, pulp, textile, ethanol and beverage manufacturers. Food and beverage industries are being the principal users of enzymes on an international basis, the total worth of the enzyme market is estimated to reach $320 million by 2020 (Rüdiger et al., 1992). The application of enzyme is an alternative for chemical based processing in the industry and it is favored as it offers the cheaper and eco-friendly alternative. There is increased energy demands have cantered global attention on the consumption of renewable

resources, mainly agricultural and horticultural residues, the main components of which are xylan, cellulose, pectin, lignin, and starch (Kaur et al., 2004).

Pectinase is a heterogeneous class of biocatalyst/enzyme. It acts on pectin and degrade it (Yadav et al., 2015).It is already reported that microorganism can able to secret pectinase, however, none of these systems described are efficacious alone to bear a commercial enterprises practicable operation. To succeed efficacious technologies for pectinase engenderment, paramount investigation has been directed towards the identification of efficient enzyme systems and process conditions. Additionally, research has been directed towards improvement of biochemical process using isolated potent organisms or tailor made organism (Nakagawa (Nakagawa et al., 2002). Since every microorganism has their own nutritional requirement, therefore, addition of proper nutrient in production media can enhance the yield of product (Dutta et al., 2017; Kumar et al., 2013). Therefore, to achieve industrial scale production of pectinase could be possible through proper design of media (Çalik et al., 2003). In the present study, an optimization study was carried out using mutant strains of *Bacillus Subtilis*MF447840.1. Thethree levels of ten factors were optimized and the size of experimentation was represented by symbolic array of L-27 (which indicated 27 experimental trials).

MATERIALS AND METHOD

Materials

Pectin was purchased from Himedia (Himedia, India). DNS reagent (Merck India), sodium potassium tartrate (SRL, India), sodium hydroxide (SRL, India), sodium sulphite (Merck, India), tri sodium citrate (SRL, India), magnesium sulphate (Merck, India), bovine serum albumin (Himedia, India), Bradford reagent (Himedia, India) were used in this study. All other chemicals were used in analytical grade commercially available in India.

Microorganism

Pectinase producing mutant strains of *Bacillus subtilis* MF447840.1 (UV-20-11/NTG-1-5/EMS-1-2) was used in entire study. The microorganism was grown on nutrient agar slants at 37 ^{o}C and pH 7.4. It was maintained by regular sub-culturing on nutrient agar slants at pH 7.4. The culture was revived by adding a loop full of pure culture into 50 ml of sterile nutrient broth (pH 7.4).

Seed culture

A 250 ml Erlenmeyer flask containing 50 ml medium was inoculated with a loop full of pure culture, which and incubated at 37^{o}C in a rotary shaker at 120 rpm at pH of 7.4 for 24 hours. The seed medium used was composed of 1 g/l of pectin, 0.5 g/l of NaCl, 0.75 g of K_2HPO_4, 0.25 gm of KH_2PO_4 and 0.5 g/l of $MgSO_4$.

Enzyme assay and determination of protein concentration

The pectinase activity was calculated as described by (Ying-xian, 2005). The concentration measurement of protein was finding by using standard Bradford 1976 method (Bradford, 1976). All experiments were done in triplicate.

Optimisation of pectinase production

Optimisation of enzyme production by Taguchi Methodology

In this study,orange peel, NaCl, K_2HPO_4, KH_2PO_4 and $MgSO_4$, inoculums percentage, temperature, pH, agitation and incubation time were considered for their effect on pectinase production from *Bacillus Subtilis* MF447840.1 (Uday et al., 2018). All the variables were investigated at three widely spaced levels. Matrix was designed with the appropriate orthogonal array (OAs) for the selected parameters and their levels using Qualitek-4 software (Nutek Inc., MI, USA). The Taguchi method involves establishing different experimental situations through orthogonal arrays (OA) which reduce experimental errors and enhance the efficiency and reproducibility of experiments. Robust design has been considered in this study because it helps minimise noise in the optimisation process and leads to a dynamic or robust experiment design (Dehnad, 1989). Planning, conducting, analysis and validation are four phases of Taguchi methodology. Each phase is separated with distinct objectives and sequencesare connected to achieve the overall optimisation process.

Design of experiments (Phase I)

In Phase I, the various factors were determined. These parameters have a critical effect on pectinase. All the variables within feasible range were investigated and variations inherent in the process do not mask the factor effect. In this study,orange peel, NaCl, K_2HPO_4, KH_2PO_4 and $MgSO_4$, inoculums percentage, temperature, pH, agitation and incubation time were considered for their effect on pectinase production. All the variables were investigated at three widely spaced levels and are shown in Table 1. In the next step, a matrix was designed with the appropriate OAs for the selected parameters and their levels. Taguchi provides many standard OAs and corresponding linear graphs for this purpose.(Taguchi, 1986). In the present study, three levels of ten factors (Table 1 and 2) were considered and the size of experimentation was represented by a symbolic array of L-27 (which indicated 27 experimental trials).

Analysis of pectinase production with selected factors and levels (Phase II)

A 2% fresh culture (OD_{550} ≈ 0.1) was inoculated into a 50 ml complex media, contained in a 250 ml Erlenmeyer flask. The media composition and incubation condition was maintained according to the design matrix (Table 2). The culture was centrifuged at 10,000 × *g* for 10 min at 4°C. The cell pellet was discarded and the supernatant was used for pectinase activity assay. All experiments were done in triplicate.

Table 1: Experimental range of the ten numerical variables studied using Taguchi methodology.

S. No.	Coded Factor	Factor	Level-1	Level-2	Level-3
1	A	Orange peel (g/l)	2	3	5
2	B	Ammonium sulfate (g/l)	1	1.5	2
3	C	Magnesium sulphate (g/l)	0.25	0.5	0.75
4	D	Phosphate ion (g/l)*	0.3	0.5	0.7
5	E	Sodium chloride (g/l)	0.25	0.5	0.75
6	F	Inoculum percentage (v/v%)	4	5	6
7	G	Temperature (°C)	35	37	39
8	H	pH	6	7	8
9	I	Agitation speed (rpm)	100	120	140
10	J	Incubation time (h)	72	96	120

Phosphate ion (0.3 g/L) is 0.30 g/l of each K_2HPO_4 and KH_2PO_4

Data analysis and prediction of performance (Phase III)

The experimental data was processed using Qualitek-4 software (Nutek Inc., MI, USA) to evaluate the influence of individual factors, multiple interaction of the selected factors, determination of optimum conditions and the process performance on pectinase activity. In the present study, S/N analysis was employed with bigger-is-better performance characteristics for all the experimental cases. In the Taguchi method, the term 'signal' represents the desirable value (mean) and the term 'noise' represents the undesirable value (SD) for the output characteristic.(Zhou et al., 2010) Therefore, the signal-to-noise (S/N) ratio is the ratio of the mean to the SD. Taguchi used the S/N ratio to measure quality characteristics deviating from the desired value. A loss function [L(y)] is developed for the deviation (Mitra, 1998) as represented by $L(y)=k\times(y-m)^2$, where k denotes the proportionality constant, m represents the target value and y is the experimental value obtained for each trial. In the case of bigger and better quality characteristics the loss function can be written as $L(y) = k\times(1/y^2)$ and the expected loss function can be represented by

$$E[L(y)] = kE(1/y^2) \quad (1)$$

Where $E(1/y^2)$ can be estimated from a sample of n as

$$\sum_{i=1}^{n}[1/y_i^2]/n \quad (2)$$

Taguchi used the S/N ratio as a performance measurement of a dynamic system to evaluate the robustness of the overall process. (Tong et al., 2004) The mathematical expression for the S/N ratio for the ''bigger is better'' case for the performance statistics that measure deviation from the target, called as mean square deviation (MSD) was given by

$$Z = -10\log(MSD) = -10\log\sum_{i=1}^{n}[1/y_i^2]/n \quad (3)$$

Validation of the experimental model (Phase IV)

In order to validate the methodology, assay experiments were further performed for pectinase production using the predicted optimised production conditions.

RESULTS AND DISCUSSION

Effects of individual factors

The effect of each factor at the assigned level used for determination of pectinase production has been listed in Table 3. It has been found that production of pectinase is mainly dependent on selected process factors. Individually all parameters are influential at Level 2. However, orange peel, and inoculum percentage are very much strong at Level 3 and Level 1 respectively.

Table 2: L_{27} OA (3^{10}) of design experiments for ten variables with actual pectinase production.

Trial No	A	B	C	D	E	F	G	H	I	J	Pectinase Production (U)		
1	1	1	1	1	1	1	1	1	1	1	246.92	249.84	252.16
2	1	1	1	1	2	2	2	2	2	2	349.23	354.62	316.39
3	1	1	1	1	3	3	3	3	3	3	201.96	185.04	193.21
4	1	2	2	2	1	1	1	2	2	2	382.94	430.52	381.77
5	1	2	2	2	2	2	2	3	3	3	351.98	360.16	345.58
6	1	2	2	2	3	3	3	1	1	1	215.97	221.23	225.32
7	1	3	3	3	1	1	1	3	3	3	279.03	255.44	288.35
8	1	3	3	3	2	2	2	1	1	1	264.78	288.35	224.78
9	1	3	3	3	3	3	3	2	2	2	272.16	279.76	247.5
10	2	1	2	3	1	2	3	1	2	3	251.59	238.15	242.84
11	2	1	2	3	2	3	1	2	3	1	319.31	337.62	311.71
12	2	1	2	3	3	1	2	3	1	2	256.25	279.76	264.42
13	2	2	3	1	1	2	3	2	3	1	356.67	346.15	354.9
14	2	2	3	1	2	3	1	3	1	2	282.52	293.61	281.35
15	2	2	3	1	3	1	2	1	2	3	357.82	353.75	346.15
16	2	3	1	2	1	2	3	3	1	2	229.98	241.07	245.75
17	2	3	1	2	2	3	1	1	2	3	286.03	279.03	284.29
18	2	3	1	2	3	1	2	2	3	1	384.09	390.52	389.35
19	3	1	3	2	1	3	2	1	3	2	417.72	422.4	451.96

20	3	1	3	2	2	1	3	2	1	3	264.42	255.1	253.33
21	3	1	3	2	3	2	1	3	2	1	244.01	248.67	239.33
22	3	2	1	3	1	3	2	2	1	3	349.07	339.72	341.49
23	3	2	1	3	2	1	3	3	2	1	341.49	329.23	337.98
24	3	2	1	3	3	2	1	1	3	2	412.54	435.46	401.88
25	3	3	2	1	1	3	2	3	2	1	296.53	288.95	295.38
26	3	3	2	1	2	1	3	1	3	2	438.97	446.55	448.89
27	3	3	2	1	3	2	1	2	1	3	249.84	242.84	246.92

Table 3: Main effects of selected factors

			S/N ratio of pectinase production (U)			
Serial No	**Coded Factor**	**Factor**	**Level 1**	**Level 2**	**Level 3**	**L2-L1**
1	A	Orange peel (g/l)	48.82	49.53	50.27	0.71
2	B	Ammonium sulfate (g/l)	48.81	50.49	49.32	1.68
3	C	Magnesium sulphate (g/l)	49.58	49.60	49.44	0.02
4	D	Phosphate ion (g/l)	49.49	49.64	49.49	0.15
5	E	Sodium chloride (g/l)	49.74	49.97	48.92	0.23
6	F	Inoculum percentage (v/v%)	50.17	49.32	49.13	-0.85
7	G	Temperature (^oC)	49.44	50.40	48.78	0.96
8	H	pH	49.86	50.08	48.69	0.22
9	I	Agitation speed (rpm)	48.32	49.60	50.70	1.28
10	J	Incubation time (h)	49.27	50.47	48.88	1.2

However, the relative effect of the factors is considered from the magnitude of difference between the average effects (L2-L1). The larger magnitude of difference between the average effects is much stronger in influence (Taguchi, 1986). It is evident from Table 3 that ammonium sulfate displays a stronger effect on pectinase activity in compare to all the other factors, followed by agitation speed, incubation time, temperature, inoculum percentage, orange peel, sodium chloride, pH, phosphate ion, and magnesium sulfate. From Table 3, it is observed that higher level of pectinase activity is found with a subsequent increase of orange peel concentration up to level 3. It is obvious that higher substrate concentration influences more bacterial growth in fermentation media and subsequent enhancement of pectinase productivity. In the case of ammonium sulphate, magnesium sulphate, phosphate ion and sodium chloride, the pectinase productivity is increased as levels of these parameters are increased from level 1 to level 2. However, further enhancement of level of these parameters decreases the productivity of enzyme in media. Since level of salts alter the osmotic pressure of the media, therefore, optimum level of these parameters are required for higher amount of growth in fermentation media.

Pectinase productivity is found to be maximum at level 1 of inoculum percentage. Further enhancement of level, the pectinase productivity is decreased. The growth of bacteria depends on the availability of nutrient in media and size of inoculum. The optimum level of inoculum size helps to utilize the media components properly. Since temperature has important role on bacterial growth, therefore, temperature has indirect relationship with productivity of pectinase. In the present case, pectinase productivity is found to be increased as temperature increased from level 1 to level 2. However, further enhancement of temperature, pectinase productivity is decreased. Since every microorganism has their own requirement of temperature for growth, therefore, level 2 of temperature is found to be optimum in the present case (Dixon & Webb, 1979; Sadana & Henley, 1988; Sode et al., 1996; Tanford, 1968). Pectinase productivity is found to be increased as level of pH

increased from level 1 to level 2. However, further enhancement of pH value (Level 3), the pectinase productivity is decreased. The variation pectinase productivity is due changes of 3D structure of protein or enzyme which are directly or indirectly involved for bacterial growth (Sadana & Henley, 1988).With an increase in agitation speed from level 1 to level 3, it is observed that pectinase productivity increases, which may be due to uniform mass or oxygen transfer. The pectinase productivity increases with an increase in incubation up to Level 2. However, pectinase productivity decreases with further increases in incubation time, which may be due to auto-degradation of pectinase.

Analysis of variance

Analysis of variance (ANOVA) was used to analyse the experimental data and to determine the variation of the result due to each factor. ANOVA reports along with the percentage of contribution of each factor are shown in Table 4. Results show that agitation speed contributed the maximum impact (26.51%) on pectinase productivity followed by ammonium sulfate (13.77%), incubation time (12.77%), temperature (12.36%), pH (V%), orange peel (9.64%), inoculum percentage (5.61%), and sodium chloride (5.467%).

Table 4: Analysis of Variance (ANOVA).

			Pectinase	production		
Factor	**DOF**	**SS**	**Variance**	**F-Ratio**	**Pure**	**Percent**
Orange peel (g/l)	2	9.43	4.7	32.81	9.14	9.64
Ammonium sulfate (g/l)	2	13.34	6.67	46.45	13.06	13.77
Magnesium sulphate (g/l)	2	0.13	0.06	0.44	0.00	0
Phosphate ion (g/l)	2	0.14	0.7	0.488	0.00	0
Sodium chloride (g/l)	2	5.47	2.74	19.05	5.18	5.467
Inoculum percentage (v/v%)	2	5.60	2.74	19.50	5.32	5.61
Temperature(^{o}C)	2	12.01	6.00	41.81	11.72	12.36
pH	2	10.01	5.00	34.86	9.73	10.26
Agitation speed (rpm)	2	25.43	12.71	88.51	25.14	26.51
Incubation time (h)	2	12.39	6.20	43.13	12.11	12.77
Other/Error	6	0.861	0.143			3.621
Total:	26	94.822				100%

It is evident from results that individually each factor influenced the production of pectinase at a certain level. However, this significant factor gives maximum yield when these factors act collectively, which may be due to the interactive effect of different factors.

Optimum process parameters

Optimum conditions of significant factors and their performance in terms of contribution for achieving high pectinase yield are shown in Table 5. It is evident from Table 5 that agitation speed plays the maximum role in pectinase productivity followed by ammonium sulfate, incubation time, temperature, orange peel, inoculum percentage, pH, sodium chloride, phosphate ion, and magnesium sulfate.

Table 5: Optimum condition for pectinase production

Coded Factor	Factor	Values	Level	Contribution for pectinase production from S/N ratio
A	Orange peel (g/l)	5	3	0.727
B	Ammonium sulfate (g/l)	1.5	2	0.95
C	Magnesium sulphate (g/l)	0.5	2	0.055
D	Phosphate ion (g/l)	0.5	2	0.102
E	Sodium chloride (g/l)	0.5	2	0.427
F	Inoculum percentage (v/v%)	1	1	0.634
G	Temperature (°C)	37	2	0.864
H	pH	7	2	0.536
I	Agitation speed (rpm)	140	3	1.157
J	Incubation time (h)	96	2	0.93
	Total contribution from all factors			6.381
	Current grand average performance			49.54
	Expected result at optimum condition			55.922

The results have found that a higher level of pectinase yield can be achieved with orange peel (5 g/L), ammonium sulfate (1.5 g/L), magnesium sulfate (0.5 g/L), phosphate ion (0.5 g/L), sodium chloride (0.5 g/L), inoculum percentage (1%), temperature (37°C), pH (7), agitation speed (140 rpm), and incubation time (96 h). The optimum conditions for expected pectinase are found where S/N ratio is 55.922 (total contribution from all the factors being found 6.381 with grand average performance of 49.54). The estimated productivity of pectinase from the S/N ratio is 625.317 U [by Eq. (3)].

Validation experiments

Fig. 1 shows the frequency distribution of current conditions along with improved conditions. It is evident from the Fig. 4.11 that pectinase productivity may be increased from 299.91 U (S/N ratio is 49.54) to 625.317 U (S/N ratio is 55.922).

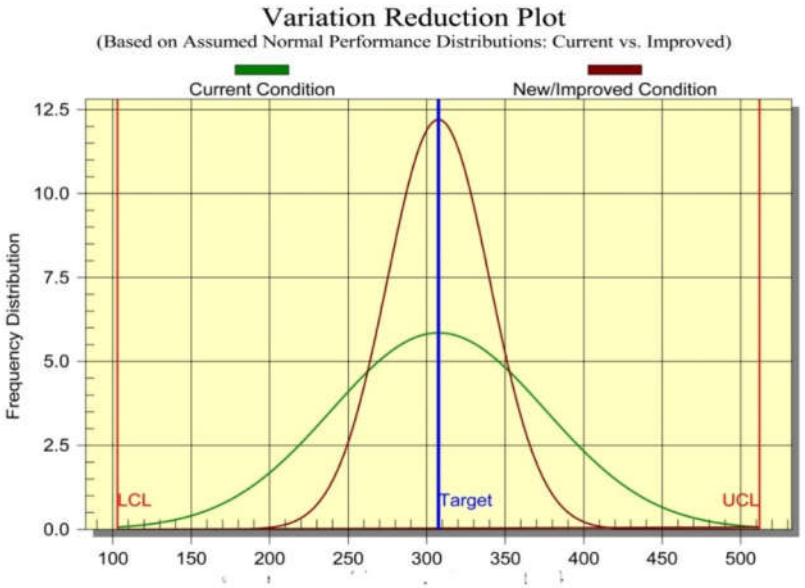

Figure 1: The frequency distribution of current conditions along with improved conditions

Overall 52.03% enhancement in the pectinase yield may be attained. Further, to validate the proposed experimental methodology, experiments employing the obtained optimised process conditioned were performed. The experimental data shows that optimised conditions enhanced the pectinase productivity up to 630.58 U (52.43%). The overall deviation of predicted results from experiment results was found to be 0.84% which was within range (Bhunia et al., 2011).

CONCLUSION

Taguchi methodology was adopted for further improvement of pectinase production through optimization of nutritional and process parameters. Ten factors were taken into consideration for their role on extracellular pectinase production. L_{27} Orthogonal Arrays (OAs) of design experiments was taken in this study and ten variables were investigated with three levels. The experimental data was processed using Qualitek-4 software (Nutek Inc., MI, USA) to evaluate the influence of individual factors, multiple interaction of the selected factors, determination of optimum conditions and the process performance on pectinase activity. The maximum pectinase productivity (630.58 U) was achieved after successful optimization using Taguchi methodology.

ACKNOWLEDGMENTS

This material is based on work supported by the National Institute of Technology, Agartala, India and Maharishi Markandeshwar (Deemed to be University), Mullana. This article does not contain any studies with human participants or animals performed by any of the authors. The authors declare that they have no conflict of interest.

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