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Optimization of polygalacturonase production by *Trichoderma harzianum* on orange peels in submerged fermentation

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Abstract: The effect of fermentation condition on polygalacturonase production by *Trichoderma harzianum*was studied using Response Surface Methodology (RSM) design. RSM revealed that the highest production of polygalacturonase reached a maximum of 145.6 U/ml. The optimum conditions for the production of the enzyme by submerged fermentation were achieved using broth medium containing 3% orange peels powder as sole carbon source. The initial pH was 6 during fermentation period of 5day at 30°C in shaking flask 150rpm. Applying the optimum conditions obtained 145.6 U./mL enzyme activity.
Keywords: Polygalacturonase, Response Surface Methodology, Production, Optimization, *Trichoderma harzianum.*

Introduction

Pectinase is a general term for enzymes, such as pectolyase, pectozyme and polygalacturonase[1] which are involved in the breakdown of pectin from a variety of plants [2].

Pectin is one of the most widely available polysaccharide in nature after cellulose, starch and chitin. The basic unit of pectin is α, D-galacturonate which is linked through α-1,4-glucosidic linkages[3]. Pectinases are classified on the basis of their preferred substrate (pectin, pectic acid oroligo-D-galacturonate), the degradation mechanism (transelimination or hydrolysis) and the type ofcleavage, random [endo-] or terminal [exo-][4]. The ability to synthesize pectinolytic enzymes is very common in groups of microorganisms[5]. Polygalacturonase are produced by numerous fungi and bacteria and also by higher plants[6-7-8-9-10]. Most of the commercial polygalacturonase are produced by *Aspergillus*species[11]. Among various Aspergilli, *Aspergillus fumigatus* is the most common in nature with a wide range of temperature tolerance and ability to produce large array of enzymes. Microbial pectinases have tremendous potential in food, beverage and textile industries as to hydrolyze the pectic substances. These are used in degumming of plant fibers, paper making, tea leaves and coffee fermentation, and in the treatment of waste waters[12]. Pectinases also help the maintenance of ecological balance by decomposition and recycling of waste plant materials[13].

Pectinases from food and food Bio products processed waste alone account to a total of one-third quarter of world's food enzyme production[14]. Pectinolytic enzymes are essential in the decay of dead plant material by nonpathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere[15-16]. These enzymes not only provide an economically viable alternative, but are also environmental friend.

Response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal conditions of a multivariable system can be determined.

In this study, *Trichoderma harzianum* strain was isolated from Tomatoes rotting. The improvement of the enzyme-productivity of the selected isolate was achieved through physiological optimization, including temperature - PH – substrate concentration - incubation period- ventilation speed.

The aim of the present study was to investigate the optimum condition of polygalacturonase production by *Trichoderma harzianum* using orange peels as a substrate in submerged fermentation

Materials and Methods

Organisms

Trichoderma harzianum strain were used (strain one isolated from Tomatoes rotting) maintained on potato dextrose agar (PDA) at 25°C for 5 days.

Preparation a spore solution:

The spore suspensions used as inoculum were obtained on PDA using the stock cultures. The incubation temperature and time for each of the steps were 25◦C and 1 week, respectively. The harvesting of the spores from the slants was done using 5ml of Tween80-water (0.02%). The spore suspension was collected in sterile falcon tube and stored at4 ◦C until the actual study. The initial spore counts and viability counts were recorded.

Substrates preparation:

We used orange peels as substrate for fungi growth and production of polygalactorunase**.** The peels were minced into pieces and hot air oven dried at 55oC until a constant weight was achieved[17]. They were ground into powder (particle size 300µm) and sealed in polyethylene bags for further use

Cultivation media and reagents used:

-potato dextrose agar medium was prepared according to manufacturer's instructions (Himedia)

-Czapek- Dox Agar: was prepared according to[18] Composed of:

Sodium nitrate 2.000
Dipotassium phosphate 1.000
Magnesium sulphate 0.500
Potassium chloride 0.500
Ferrous sulphate 0.010
Agar 15.000

Final pH (at 25°C) 6.2and citrus pecctin(10 g/l) were used As a substitute for sucrose. These components were dissolved in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.

Submerged fermentation

Mandels medium[19] with the following composition

0.3g/l urea
1.4 g/l (NH4)2SO4
2.0 g/l KH2PO4,
0.4g/l CaCl2·2H2O,
0.3 g/l MgSO4·4H2O,
0.75 g/l peptone,
0.25g/l yeast extract,

5 mg/l $FeSO_4 \cdot 7H_2O$
1.6 mg/l$MnSO_4 \cdot 4H_2O$,
1.4 mg/l $ZnSO_4 \cdot 7H_2O$
20 mg/l $CoCl_2 \cdot 6H_2O$, orange peels were used as fermentation substrate.

A volume of 50ml of the basal medium was added into 250ml Erlenmeyer's flasks and labeled appropriately. The flasks were autoclaved at 121oC for 15minutes. Each flask was inoculated with a suspension containing 106 cells/ml of the selected pectinolytic isolates. The flasks were then incubated in a rotary shaker.

Enzyme Assay using Spectrophotometer

After inoculation of the different media with the inocula, the crude enzyme was extracted by centrifugation at 8500rpm for 20min. The resultant extract was used for enzyme assay. The filtrates obtained after biomass separation after incubation was stored at 4oC for enzyme assay Polygalacturonase activity was determined by standard colorimetric method using polygalacturonic acid as substrate. Polygalacturonase (PG) activity was determined by measuring the release of reducing groups from pectin using the 3,5-dinitrosalicyclic acid reagent assay[20]. The reaction mixture containing 0.8ml 1% citric pectin in 0.2M acetate buffer, pH 5.0 and 0.2ml of crude enzyme, was incubated at 40oC for 10 minutes using a modified method of [21]. One unit of enzyme activity was defined as the amount of enzyme required to release one micromole of galacturonic acid per ml per minute under standard assay conditions.

Optimization of polygalacturonase production from *Trichoderma harzianum* using orange peels as substrate

The study was carried out Using Advanced design experiments (Response Surface Methology) Using Minitap Optimization Method To study the effect of each factor separately and the impact of factors Interacting with each other.

Experience design:

Optimal conditions for Polygalacturonase production have been studied (temperature -pH – substrate concentration - incubation period- ventilation speed), we studied each variable at three levels as follows (+1, 0, -1):

Effect of incubation period: A group of flasks containing basal medium were inoculated for (3-5-7) day incubation periods.

Effect of incubation temperature: A set of flasks containing basal medium was incubated at different temperatures (20, 30, 40°C).

Effect of carbohydrate: orange peel was added in basal medium at different concentrations (1% -3% -5%).

Effect of initial pH: Different flasks containing basal medium with initial pH values (4-6-8) were inoculated with the tested organism and incubated.

Effect of ventilation speed: Different speeds of shaking flask were used (100-150-200)rpm.

Statistical analysis

Experimental design RSM included the effect of orange peel concentration as a carbon source, , the effect of initial pH, the effect of fermentation temperature, and the effect of incubation periods, the effect of ventilation speed.

This design contains 32 experimental plots. Experimental results of polygalacturonase production by a complete 5-factor, with 3 replications of the central point.

Results and Discussion

Table (1) shows the results of the enzymatic activity produced by *Trichoderma harzianum* after optimizing medium conditions using the statistical test (RSM) as adopted statistical design that contains orange peelsconcentration, incubation period, pH, the temperature of fermentation medium and. ventilation speed

Table (1) Activity values for each treatment for the *Trichoderma harzianum* isolate:

Temperature C°	pH	Aeration speed rpm/m	Substrate concentration%	Incubation time Day	Enzyme acyivity U./mL	Blocks
20	4	100	1	7	7.77	1
20	4	100	1	3	12.85	2
20	8	100	1	3	16.30	3
40	8	100	1	7	43.28	4
20	4	200	1	3	7.81	5
40	4	200	1	7	4.66	6
20	8	200	1	7	41.69	7
40	8	200	1	3	19.87	8
30	4	100	5	3	334.11	9
40	4	100	5	7	15.94	10
20	8	100	5	7	177.13	11
40	8	100	5	3	3.67	12
20	4	200	5	7	77.47	13
40	4	200	5	3	8.88	14
20	8	200	5	3	174.82	15
40	8	200	5	7	15.21	16
20	6	150	3	5	205.29	17
40	6	150	3	5	7.753	18
30	4	150	3	5	186.09	19
30	8	150	3	5	107.3	20
30	6	100	3	5	177.93	21
10	6	200	3	5	89.64	22
30	6	150	1	5	128.13	23
30	6	150	5	5	77.73	24
30	6	150	3	3	92.51	25
30	6	150	3	7	122.20	26
30	6	150	3	5	151.46	27
30	6	150	3	5	150.75	28
30	6	150	3	5	153.19	29
30	6	150	3	5	149.62	30
30	6	150	3	5	149.94	31
30	6	150	3	5	147.15	32

The results in Table (1) clearly showed that there were differences in the enzymatic activity values in all experimentsAccording to temperature, PH, substrate concentration, incubation period and ventilation speed in fermentation medium.

These results were close to the results of[22] where the optimal polygalacturonase production Increased in 50-55^{0}c. In addition, pH demonstrated a very important effect on the growth of microorganisms, as it effected the solubility of nutrients in the culture, and was reflected on the growth of fungi and the enzyme production. These results were in conformity with the findings of[23] in a study of polygalacturonase production where the optimal pH for producing PG from *Trichoderma harzianum* was 6.

Optimal condition for the enzyme production by the statistical test RSM:

Optimal conditions for the production of polygalacturonase produced from *Trichoderma harzianum* after application of the statistical test RSM were indicated in (Figure 1). The maximum production of enzyme was 147.062 U./mL at a temperature of 30°C, pH 6, incubation time 5days, orange peels concentration 3% andventilation speed 150 rpm.

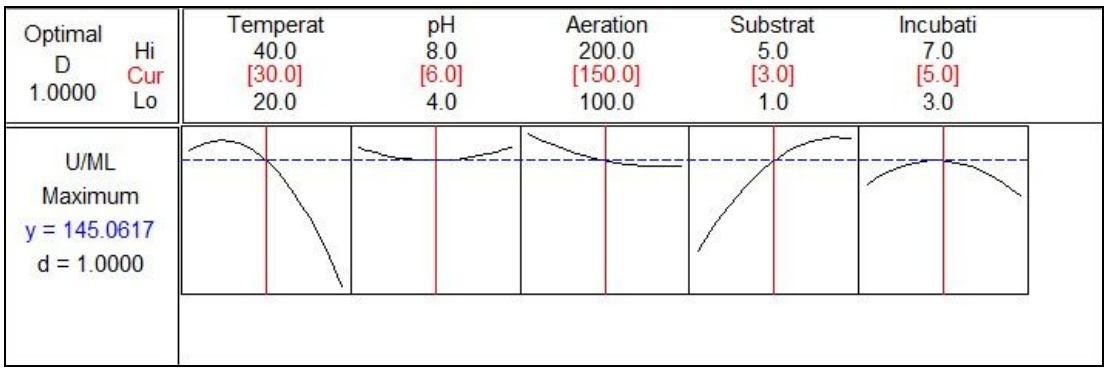

Figure 1: The optimal conditions for polygalacturonase production from *Trichoderma harzianum* using statistical program RS

The statistical analysis showed that there was a significant interaction effect between pH and the incubation time at a confidence level of 5%.

Results of the RSM statistical test for glucose polygalacturonase production

Table (2) showed the effect of the studied factors (the linear terms, the squared terms, and the interactions) on *polygalacturonase* production. The small *P* values for the temperature , substrate concentration and ventilation speed($P < 0.05$) indicated that there were significant linear effects of each of these two variables on enzyme production, while pH value andincubation periodhad no linear effects on enzyme production. On the other hand, the small *P* values for the effect of squared terms of incubation time, substrate concentration and temperature ($P < 0.05$) suggested that there was curvature in the response surface. Furthermore, the interactions between the incubation time and temperature, and between temperature and pH were significant ($P < 0.05$). Consequently, the following equation could be deduced:

$$Y = 145.06 - 62.64X_1 + 0.44X_2 - 14.67X_3 + 51.96X_4 - 5.70X_5 + 18.99X_1X_2 + 30.18X_1X_3$$
$$- 44.37X_1X_4 + 31.77X_1X_5 + 13.67X_2X_3 - 30.39X_2X_4 + 16.09X_2X_5 - 36.31X_3X_4 - 0.78X_3X_5$$ Where
$$- 38.14X_4X_5 - 53.21X_1^2 + 12.13X_2^2 + 9.55X_3^2 - 31.64X_4^2 - 27.21X_5^2$$

represent:
A: constant
f: Variablesfactors‹e ‹d ‹c ‹b
k: factors squarevariables‹j ‹i ‹h ‹g
u: Correlated variables factors ‹t ‹s ‹r ‹q ‹p ‹o ‹n ‹m ‹l
Statistical analysiswasat the level0.05.

Table (2) Estimated Regression Coefficients for Enzyme activity:

Term	Coef	SE Coef	T	P
Constant	145.062	5.298	27.38	0
Temperature	-62.642	7.3	-8.581	0
pH	0.441	4.801	0.092	0.927
Aeration speed	-14.668	4.951	-2.962	0.004
Substrate concentration	51.957	6.496	7.998	0
Incubation time	-5.696	4.801	-1.186	0.239
Temperature*Temperature	-53.123	11.557	-4.597	0
pH*pH	12.127	11.277	1.075	0.286
Aeration speed*Aeration speed	9.551	13.138	0.727	0.469
Substrate concentration*Substrate concentration	-31.636	11.277	-2.805	0.006
Incubation time*Incubation time	-27.213	11.277	-2.413	0.018
Temperature*pH	18.99	8.172	2.324	0.023
Temperature*Aeration speed	30.185	8.214	3.675	0
Temperature*Substrate concentration	-44.372	6.045	-7.34	0
Temperature*Incubation time	31.775	8.172	3.888	0
pH*Aeration speed	13.674	5.161	2.65	0.01
pH*Substrate concentration	-30.39	7.133	-4.261	0
pH*Incubation time	16.094	5.161	3.119	0.003
Aeration speed*Substrate concentration	-36.307	7.133	-5.09	0
Aeration speed*Incubation time	-0.78	5.161	-0.151	0.88
Substrate concentration*Incubation time	-38.14	7.133	-5.347	0

Conclusions

The isolate *Trichoderma harzianum* was able to produce polygalacturonase and the optimum conditions for production of the enzyme were achieved on broth medium containing 3% of orange peels as a sole carbon source and inducer for enzyme production, with an initial pH of 6 during an incubation period of 5 daye at 30°C in shaking flask by Aeration speed 150 rpm. These optimum conditions for enzyme activity attained 145.062 U/ml. This factor exhibited significant squared effects on enzyme production at a confidence level of 5%.

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