

Polygalacturonase Production by *Sclerotinia sclerotiorum*, Causal Agent of Canola Stem Rot: Parameter Optimization Using Taguchi Approach

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Abstract: Submerged culture conditions for polygalacturonase production by *Sclerotinia sclerotiorum* (MR5) were optimized by Taguchi orthogonal array experimental design methodology. This approach facilitates the study of interaction of a large number of variables spanned by factors and their settings with a small number of experiments leading to considerable saving in time and cost for the process optimization. The objective of the current research was to determine the significant parameters on the production of polygalacturonase in a submerged culture of *S. sclerotiorum*. The process variables were pH, pectin as carbon source and inducer, incubation time and temperature. The liquid medium also included mineral salts as in Pectic Zymogram (PZ) medium. Polygalacturonase activity was calculated at the time of maximal extracellular enzyme activity. The optimal levels of the different factors for polygalacturonase production were 15 g l⁻¹ pectin, pH 5 and 4 days of incubation time at 25°C. Temperature was the most important factor in the enzyme production with 54.6% contribution, whereas pectin had a minimal contribution (3.19%).

Key words: *Sclerotinia sclerotiorum* • polygalacturonase • phytopathogen

INTRODUCTION

Phytopathogen *Sclerotinia sclerotiorum* is worldwide in distribution and is pathogenic to more than 480 plant species including oilseed crops [1]. Infection of oilseed plants can occur any time after seedling emergence. *Sclerotinia* diseases cause serious yield losses of oilseed crops including sunflower, soybean and canola [2]. Stem rot is one of the most significant diseases in soybean and canola [3].

Many phytopathogenic fungi including *S. sclerotiorum* produce polygalacturonase (PG) enzymes which are thought to play an important role during the early stages of infection to degrade the pectin component of plant cell walls [4]. Polygalacturonases are induced by pectin and repressed by the presence of carbon catabolites, such as glucose [5]. pH also influences the expression of this enzyme [6, 7]. Specific activity, substrate specificity and pH optimum may be advantageous for a fungal pathogen [8].

Taguchi's method has been used to generate enough process information to establish the screening and optimal conditions of parameters for particular process

using a minimum number of experiments possible [9]. A few reports are available on the application of Taguchi's method in the field of biotechnology [10-13]. Taguchi's method was used successfully to optimize the reaction variables and their ranges for PG production. The basic principle of this method serves as screening filters which examine the effects of process variables and identify those factors which have major effects as process using a single trial with a few experiments [13, 14].

Environmental factors such as carbon source, temperature and pH and operating parameter such as incubation time could affect the production of PG from *S. sclerotiorum* in submerged culture. The objective of the current study was to determine the significant parameters on the production of PG in a submerged culture of *S. sclerotiorum* to study the PG interaction with its inhibitor proteins.

MATERIALS AND METHODS

Microorganisms and cultural conditions: *Sclerotinia sclerotiorum* (MR5), causal agent of canola stem rot, was isolated from infected canola plants in Dashte-Naz from

Table 1: Taguchi's experimental design matrix and corresponding polygalacturonase production by *S. sclerotiorum*

Trial No.	Levels			
	pH	Incubation time	Pectin	Temperature
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	1	4	4	4
5	2	1	2	3
6	2	2	1	4
7	2	3	4	1
8	2	4	3	2
9	3	1	3	4
10	3	2	4	3
11	3	3	1	2
12	3	4	2	1
13	4	1	4	2
14	4	2	3	1
15	4	3	2	4
16	4	4	1	3

Table 2: Variable (factors) and their levels employed in Taguchi method

Factors	Level 1	Level 2	Level 3	Level 4
pH	4.0	4.5	5.0	5.5
Incubation time (day)	2.0	4.0	6.0	8.0
Pectin (g lit ⁻¹)	7.5	10.0	12.5	15.0
Temperature	15.0	20.0	25.0	28.0

Mazandaran province of Iran. The fungus was propagated on Potato Dextrose Agar (PDA) and subcultured as needed. The stock culture was stored on agar (1.5%) slants of MY medium (2% malt extract, 0.2% yeast extract, 1% maltose).

Experimental design: To reduce experimental errors and to enhance the efficiency and reproducibility of laboratory experiments Taguchi has established orthogonal arrays to describe a large number of experimental situations mainly. The symbolic designation of these arrays indicates the main information on the size of the experimentation, e.g. M16 has 16 trials. The total degree of freedom available in an orthogonal arrays is equal to the number of trials minus one. Each column consists of a number of conditions depending on the levels assigned to each factor. In the present study all four columns are assigned with different factors as indicated in Table 1. Each of these factors is assigned with four levels (Table 2). Therefore, these factors have four level 1, four level 2 and four level 3 and four level 4 conditions. Table 1 shows the layout of the

M16 orthogonal arrays used in the present study. All the combination experiments using the assigned parameter values were conducted using PZ media containing 2.64 g (NH₄)₂ SO₄, 0.34 g KH₂PO₄, 0.14 g MgSO₄.7H₂O, 10 g Citrus pectin, 1 lit. dH₂O. pH adjusted to 4-5.5 and incubated in an orbital shaker (150 rpm) [15]. After appropriate time of incubation the liquid culture filtrate was obtained (as crude enzyme), by Whatman filter paper No.1 and stored at -20°C until using for enzyme assay or protein measurement.

Qualitek-4 software for automatic design and analysis of Taguchi experiments was used to study the following objectives of the analysis.

- Identification of the individual influence of each factor.
- Determination of the optimum condition and
- Estimation of performance at the optimum condition.

Polygalacturonase assay: The fungal isolate was grown on 10 ml of the media in 25 ml Erlenmeyer flasks and after incubation at 26°C the mycelium was removed by vacuum filtration and the filtrate was clarified by centrifugation at 14000g for 5 min at 4°C. The supernatant was collected and placed into another eppendorf tube for enzyme assay. Polygalacturonase (PG) activity was assayed by measuring the release of the reducing groups using the Somogi assay with Nelson's arsenomolibdate reagent [16]. The reaction mixture, containing 0.9 ml of 25% polygalacturonic acid in 25 mM citrate-phosphate buffer pH 4.5 and 0.1 ml of enzyme solution, was incubated at 40°C for 20 min. One unit of PG activity was defined as the amount of enzyme that releases 1 µmol of galacturonic acid per minute.

This study was carried out in Plant pests and Diseases Research Institute and National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, I.R. of Iran.

RESULTS

Submerged fermentation experiments studies with the designed experimental condition showed significant variation in the polygalacturonase activity from *S. sclerotiorum* (MR5) (Fig. 1). Production levels were found to be very much dependent on the culture conditions. An M16 orthogonal experimental design was used to investigate four different culture components, pH, incubation time, amount of pectin as carbon source/inducer and temperature. The experiments were conducted using four levels for each factor. Statistical

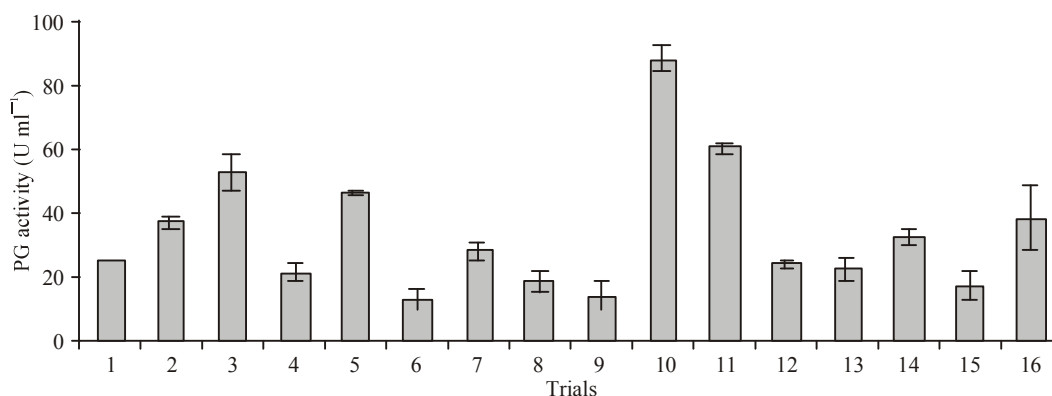


Fig. 1: Polygalacturonase production by *Sclerotinia sclerotiorum* (MR5) in a submerged culture using Taguchi orthogonal array (Three replicates)

Table 3: Main effect of factors or average of obtained results (as U ml⁻¹), in which each factor is at a given level. For description of "levels", refer to Table 1

Factors	Level 1	Level 2	Level 3	Level 4
pH	33.708	26.125	46.020	27.125
Incubation time (days)	26.541	42.104	39.416	24.916
Pectin (g l ⁻¹)	33.916	30.458	29.208	39.395
Temperature (°C)	26.875	34.375	55.937	15.791

Table 4: Estimation of severity index for different parameters (factors)

Interaction factor pairs	Columns	SI (%)	Co.	Levels
pH×incubation time	1×2	30.01	3	[3, 2]
Pectin×temperature	3×4	14.69	7	[4, 3]
pH×pectin	1×3	14.3	2	[3, 4]
pH×temperature	1×4	11.85	5	[3, 3]
Incubation time×temperature	2×4	5.12	6	[2, 3]
Incubation time×pectin	2×3	2.28	1	[2, 4]

Columns-represent the column locations to which the interacting factors are assigned, SI-Interaction SI presents 100% of SI for 90 degrees angle between the lines while, 0% SI for parallel lines. Co.-shows the column that should be reserved if this interaction effect has to be studied, Levels-indicate the factor levels desirable for the optimum conditions

analysis of the collected data pointed out that the optimal levels of the different factors for polygalacturonase production were pH 5, 4 days of incubation and 15 g l⁻¹ it pectin and incubation temperature at 25°C (Trial 10). A maximal production of 87 U ml⁻¹ was reached in these conditions after (Fig. 1).

The average affect of the factors at the assigned levels on the polygalacturonase production by *S. sclerotiorum* (MR5) was shown in Table 3. This table shows the influence of four individual factors (pH, incubation time, pectin and temperature) on the polygalacturonase yield. Individually at level stage,

temperature has the highest affect in level 3 with 55.93 U ml⁻¹ whereas pectin as carbon source/inducer, pH and incubation time show their higher affects in level 4 with 39.39 U ml⁻¹, level 3 with 46.02 U ml⁻¹ and level 2 with 42.1 U ml⁻¹, respectively on the polygalacturonase yield (Table 3). Culture temperature condition is one of the important parameter in fungal cultivation.

However, when interactions of different factors were calculated (Table 4), it appears that incubation time and pH interaction showed highest interaction severity index (SI) with 30.01%, while the severity index percentage for incubation time versus carbon source was only 2.28. These results suggested that the influence of one factor on polygalacturonase production was dependent on the condition of the other factors in optimizing polygalacturonase production process parameters.

The percentage contribution of each factor is shown in ANOVA table (Table 5). The last column of this ANOVA table indicates the influence of each factor. Incubation temperature was the most significant factor for polygalacturonase production. While incubation time and pH were observed to exert 14 and 15.3% contribution, the amount of pectin showed to have the least percentage of contribution on the yield of polygalacturonase production at their individual levels.

Optimum conditions and their performance in terms of contribution for achieving higher polygalacturonase yield are shown in Table 6. The presented data show that culture temperature has the highest role (22.7 U ml⁻¹) in the enzyme production, while the pH, incubation time and pectin showed to have 12.7, 8.8 and 6.1 U ml⁻¹ contribution, respectively. The expected result at optimum condition was 83.7 U ml⁻¹ with total contribution from all

Table 5: Analysis of variance (ANOVA)

Factors	DOF	Sums of squares	Variance	f-Ratio	Pure sum	Percentage contribution (%)
pH	3	3019.024	1006.341	19.897	2867.298	15.378
Incubation time	3	2770.445	923.481	18.259	2618.718	14.045
Pectin	3	748.131	249.377	4.930	596.405	3.198
Temperature	3	10337.070	3445.690	68.129	10185.344	54.628
Other/Error	35	1770.137	50.575			12.751
Total	47	18644.810				100.000

Table 6: Optimum conditions suggested by statistical calculations after performing the tests

Factors	Level desc.	Level	Contribution
pH	5	3	12.776
Incubation time	4	2	8.859
Pectin	15	4	6.151
temperature	25	3	22.692
Total contribution from all factors			50.478
Current grand average of performance			33.244
Expected results at optimum condition			83.722

the factors being 50.4 U ml⁻¹ and grand average performance of 33.2 U ml⁻¹. Comparison of four predicted parameters (pH, incubation time, pectin and temperature) from optimum condition with those of trial 10 from orthogonal array revealed that they have the same level of these four factors.

DISCUSSION

Stem rot of canola (*Brassica napus*) caused by *S. sclerotiorum* is one of the serious and most destructive plant diseases [3, 17]. The disease was found in all canola fields in Iran causing up to 54.4% annual losses [18]. Polygalacturonases which are known to be pathogenic factor of fungal pathogen are secreted by these fungi during the early phases of tissue infection [19-21].

PGIPs are important elements of plant defense mechanisms against fungal pathogens due to their capacity to interact with fungal PGs [22, 23]. A direct consequence of the PG-PGIP interaction is the reduction of fungal growth and tissue maceration by phytopathogenic fungi [24, 25].

In this study PG secretion from *S. sclerotiorum* (MR5) should be optimized for production of this enzyme to study PGIP-PG interaction. Environmental conditions can affect protein production and secretion of pectolytic enzymes in various organisms [5, 26, 27]. Temperature, incubation time, pH and level of pectin as carbon source are the major environmental factors affecting PG production in fungal pathogens [28-30]. These factors could affect the production of PG from

S. sclerotiorum in submerged culture. The method of studying one variable at a time, while keeping all others at a predetermined level not only is very inefficient in many cases and also a time consuming technique but also has the limitation of ignoring the importance of interaction of various parameters [31-32]. Taguchi approach of orthogonal array experimental design for process optimization, involving a study of given system by a set of independent variables (factors) over a specific region of interest (levels) by identifying the influence of individual factors, establish the relationship between variables and also the performance at the optimum levels obtained.

By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process can be characterized. The characteristics can be controlled such that a lower or a higher value in a particular influencing factor produces the preferred result. Thus, the levels of factors, to produce the best results can be predicted [33, 34]. Any individual factor may interact with the other factors creating the possibility of presence of interactions. This kind of interaction is possible in Taguchi design of experiment. Estimated interaction severity index (SI) of the factors under study helps to know the influence of two individual factors at various levels of the interactions [12, 35, 36].

In this experiment increase in concentration of pectin as carbon source and inducer up to level 4, has resulted in increase in enzyme production. Increase in pH has resulted in higher polygalacturonase expression up to level 3 (pH 5) and subsequent increase resulted in decrease in the polygalacturonase yield. While in cases of incubation time and temperature the polygalacturonase yield was higher up to level 3; subsequent increase in the incubation time and temperature (level 4) reduced the yield.

The influence of these four factors on polygalacturonase production show that temperature plays a more significant role than the other selected parameters (e.g. 54.02% in compared with 15.38, 14.04 and 3.19% for pH, incubation time and pectin, respectively (Table 5).

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