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Determination of Culture Condition for Polygalacturonase Production by *Rhizoctonia solani* AG2-2, Causal Agent of Root Rot in Sugar Beet

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Abstract: The objective of the current research was to determine the significant parameters on the production of PG in a submerged culture of *R. solani* AG2-2 to study PGIP-PG interaction. Taguchi method was applied to evaluate the significant parameters for PG production. The process variables were pH (4, 4.5, 5 and 5.5), pectin as carbon source and inducer (7.5, 10, 12.5 and 15 g L^{-1}) and incubation time (2, 4, 6 and 8 days). The liquid medium also included mineral salts as in Pectic Zymogram (PZ) medium. Polygalacturonase activity was determined spectrophotometrically at 500 nm and productivity was calculated at the time of maximal extracellular enzyme activity. Pectin concentration was the most important factor in the enzyme production (34.8% contribution), whereas pH had a minimal contribution (16.9%). The optimal levels of the different factors for PG production were 12.5 g L^{-1} pectin, pH 4.5 and 6 days of incubation time at 26°C. A maximal productivity of 115.5 U mL^{-1} was reached in these conditions.

Key words: *Rhizoctonia solani*, polygalacturonase, phytopathogen

INTRODUCTION

The soil fungus *Rhizoctonia solani* is one of the most important plant pathogenic fungi. The pathogen has worldwide distribution and infects a very wide range of plant species. Isolates show considerable variation in morphological and pathogenic characteristics but can be usefully divided into Anastomosis Groups (AG) that show some correlation with pathogenicity (Carling, 1996). Root rot disease which is caused by *R. solani* AG2-2, causes major losses in sugar beet.

Many phytopathogenic fungi including *R. solani* 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 (Hahn *et al*., 1989). Pectin is a complex polysaccharide, which is broken down by a suite of enzymes, including PG with endo and exo modes of action (Berger *et al*., 2000). These enzymes not only provides the fungus with a nutrient source for growth, but can facilitate the degradation of other cell wall components by fungal enzymes (Hahn *et al*., 1981). Endo-PGs of *R. solani* appear to contribute to the fungal invasion and degradation of host tissues. In general PGs are induced by pectin and repressed by the presence of carbon catabolites, such as glucose (Annis and Goodwin, 1997). pH also influences the expression of this enzyme (Wubben *et al*., 2000; Di-Pietro and Roncero, 1998).

Most fungi produce multiple isozymes that differ in their enzymatic properties, molecular weight and regulation (D'Ovidio *et al*., 2004). The multiplicity of PG isoforms may reflect the complexity of the pectin molecule in plant cell walls and the need for enzymes capable of cleaving the homogalacturonan back bone in variety of structural contexts. For these reason, specific activity, substrate specificity and pH optimum may be advantageous for a fungal pathogen (D'Ovidio *et al*., 2004).

Inhibitors of fungal enzymes that degrade plant cell walls have been proposed to be part of the plant defenses that limit the development of disease symptoms caused by microbial pathogens (Cervone *et al*., 1989; Stotz *et al*., 1993, 1994). Polygalacturonase inhibitor proteins (PGIPs) are glycoproteins located in plant cell walls that specifically inhibit fungal PGs, especially those with mixed endo/exo functions (Cook *et al*., 1999).

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 (Taguchi, 1986). A few reports are available on the application of Taguchi's method in the field of biotechnology (Jeney *et al*., 1999; Cobb and Clarkson, 1994; Han *et al*., 1998; Han and Rhee, 1998). Taguchi's method was used successfully to optimize the reaction variables and their ranges for PG production. The basic

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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 (Han and Rhee, 1998). In Taguchi's method, factors are arranged into an orthogonal array. The properties of an orthogonal array are such that, between each pair of columns each combination of levels (of variables) appear an equal number of times. Due to orthogonality of the layout, the effects of the other factors will be balanced and give a relative value representing the effects of a level compared with the other levels of a given factor. Orthogonal array experiments minimize the number of test runs while keeping the pair wise balancing property (Byrne and Taguchi, 1986).

Environmental factors such as carbon source and pH and operating parameter such as incubation time could affect the production of PG from *R. solani* 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 *R. solani* AG2-2 to study PGIP-PG interaction.

MATERIALS AND METHODS

Fungal isolate and growth conditions: *Rhizoctonia solani* (AG 2-2), causal agent of root rot in sugar beet, was kindly supplied by Professor Banihashemi, Mycology Laboratory, Department of plant Pathology, College of Agriculture, Shiraz University, Shiraz, Islamic Republic of Iran. The fungus was propagated on Potato Dextrose Agar (PDA) and subcultured as needed.

Experimental design: Taguchi has established orthogonal arrays to describe a large number of experimental situations mainly to reduce experimental errors and to enhance the efficiency and reproducibility of laboratory experiments. 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 three columns are assigned with different factors as indicated in Table 1. Each of these factors is assigned with four levels. Therefore, these factors have four level 1, four level 2 and four level 3 and four level 4 conditions. Table 2 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

Table 1: 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 L^{-1})	7.5	10.0	12.5	15.0

Table 2: Taguchi's experimental design matrix and corresponding polygalacturonase production by *R. solani* AG2-2

	Levels			
Trial No.	pH	Incubation time	Pectin	PG activity (U mL^{-1})
1	1	1	1	47.3
2	1	2	2	64.6
3	1	3	3	102.0
4	1	4	4	89.6
5	2	1	2	91.0
6	2	2	1	94.0
7	2	3	4	97.3
8	2	4	3	91.3
9	3	1	3	84.0
10	3	2	4	98.0
11	3	3	1	86.0
12	3	4	2	59.3
13	4	1	4	78.0
14	4	2	3	93.0
15	4	3	2	78.6
16	4	4	1	53.0

media containing 2.64 g $(NH_4)_2$ SO_4, 0.34 g KH_2PO_4, 0.14 g $MgSO_4$. $7H_2O$, 10 g Citrus pectin, 1 L. dH_2O. pH adjusted to 4-5.5 and incubated in an orbital shaker (150 rpm)(Sweetingham *et al*., 1986). 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 14000 g 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 (Collmer *et al*., 1988). 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 μ mol of galacturonic a cid per minute.

This study was carried out in Plant Biotechnology Laboratory in National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, Islamic Republic of Iran.

RESULTS

Taguchi method was used for the design of an experimental strategy aimed at optimizing polygalacturonase production from a *R. solani* AG2-2 (R16) isolate. An M16 arthogonal experimental design was used to investigate three different culture components, pH, amount of pectin as carbon source/inducer and incubation time. The experiments were conducted using four levels for each factor. Statistical analysis of the collected data pointed out that the optimal levels of the different factors for polygalacturonase production were 12.5 g L^{-1} pectin, 6 days of incubation and pH 4 (Trial 3). In this condition, a maximal production of 102 U mL^{-1} was reached after incubation at 26°C (Table 2). Production levels in submerged fermentation experiments were found to be very much dependent on the culture conditions.

The average affect of the factors at the assigned levels on the polygalacturonase production by *R. solani* AG2-2 (R16) was shown in Fig. 1. This Figure shows the influence of three individual factors (pH, incubation time and pectin) on the polygalacturonase yield. Individually at level stage, pH has highest affect in level 2 with 93.4 U mL^{-1} where as both pectin as carbon source/inducer and incubation time has higher affects in level 3 with 92.5 U mL^{-1} and 91 U mL^{-1}, respectively on the polygalacturonase yield. Culture pH condition is one of the important parameter in fungal cultivation.

However, when interactions of different factors were calculated (Table 3), it appears that both incubation time and pectin (at level 3; column 1) interaction showed highest interaction severity index (SI) with 66.76% followed by pH and pectin (at levels 1 and 3, column 2) with 18.59% and pH/incubation time (at levels 1 and 3, column 3) with 13.1%.

In order to determine the control factor that significantly affects the quality characteristic, analysis of variance calculations were done through Qualitek-4 software. Based on the ANOVA results computed in Table 4, it can be seen that pectin concentration exerts the most influence in polygalacturonase production (whereby the percentage contribution is 34.8%), followed by incubation time and pH.

The presented data shows that in Table 5 pH, pectin and incubation time have significant role in the enzyme production. The expected result at optimum condition was 113.6 U mL^{-1} with total contribution from all the factors being 31.9 U mL^{-1} with grand average performance of 81.7 U mL^{-1}. Taguchi design of experiment, used for culture condition optimization for production of polygalacturonase from *R. solani* AG2-2 (R16), revealed the effect of varying factors. Three studied factors in culture medium (pH, incubation time and pectin) seem to have strong influence on the polygalacturonase expression.

The higher levels of polygalacturonase activity can be achieved with obtained optimization culture conditions: pH 4.5; incubation time, 6 days; and pectin, 12.5 g L^{-1} (Table 5). It is evident from Table 5, that upon considering the optimum culture condition from the experiments designed, the polygalacturonase yield can be

Table 3: Estimation of severity index for different parameters (factors)

	Interaction factor pairs	Columns	SI (%)	Co.	Levels
1	Time×pectin	2×3	66.76	1	(3,3)
2	pH×pectin	1×3	18.59	2	(1,3)
3	pH×time	1×2	13.10	3	(1,3)

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

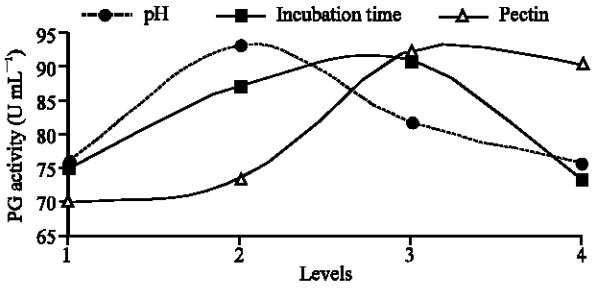

Fig. 1: Main effect of factors or average of obtained results (U mL^{-1}), in which each factor is at a given level. For description of levels, refer to Table 1

Table 4: Analysis of variance (ANOVA) results

Factors	df	Sum of squares	Variance	f-ratio	Pure sum	Percent
pH	3	2485.759	828.586	10.177	2241.508	16.952
Incubation time	3	2795.395	931.798	11.444	2551.144	19.294
Pectin	3	4846.911	1615.637	19.843	4602.660	34.810
Other/Error	38	3093.851	81.417			28.944
Total	47	13221.919				100.000

Table 5: Optimum conditions suggested by statistical calculations after performing the tests

Factors	Level Desc.	Level	Contribution
pH	4.5	2	11.708
Incubation time	6.0	3	9.291
Pectin	12.5	3	10.875
Total contribution from all factors			31.873
Current grand average of performance			81.708
Expected results at optimum condition			113.582

increased from 102 to 113.6 U mL^{-1} i.e., overall 11.4% enhancement in the enzyme production can be achieved. Further to validate the proposed experimental methodology, submerged experiments were performed for polygalacturonase production by employing the obtained optimized culture conditions (Table 5). The experimental data showed an enhanced polygalacturonase yield of 115.5 U mL^{-1} from 102 U mL^{-1} (13% improvement in polygalacturonase production with the modified culture conditions.

DISCUSSION

Root rot disease which is caused by *R. solani* AG2-2, causes major losses in sugar beet. Polygalacturonase enzymes are important virulence factors for pathogenic fungi (TenHave *et al*., 1998; Isshiki *et al*., 2001). During parasitic growth, the majority of fungal pathogens need to produce PGs to degrade the homogalacturonan component of plant cell wall (Oeser *et al*., 2002; Rodriguez-Palenzuela and Burr, 1991; Huang and Allen, 2000). PGIPs are important elements of plant defense mechanisms against fungal pathogens due to their capacity to interact with fungal PGs (Salvi *et al*., 1990; Favaron *et al*., 1997).

In this study PG secretion from *R. solani* (AG2-2) should be optimized for production of this enzyme to study PGIP-PG interaction. Enviromental conditions can affect protein production and secretion of pectolytic enzymes in various organisms (MacKenzie *et al*., 1993; Condemine and Robert-Baudouy, 1995; Annis and Goodwin, 1997). Incubation time, pH and level of pectin as carbon source are the major environmental factors affecting PG production in fungal pathogens (Chilosi and Magro, 1998; Yakoby *et al*., 2000; Nair *et al*., 2004). These factors could affect the production of PG from *R. solani* 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 (Mason *et al*., 1989; Stowe and Mayer, 1999). 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. In this methodology, the desired design is sought by selecting the best performance under conditions that produces consistent performance leads to a more fully developed process (Venkata-Dasu *et al*., 2003).

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 (Sreenivas-Rao *et al*., 2004; Chang *et al*., 2006).

Understanding the interaction between two factors gives a better insight into the overall process analysis. 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 (Han *et al*., 1998; Venkata-Dasu *et al*., 2003; Koo *et al*., 2006).

In this experiment increase in concentration of pectin as carbon source and inducer up to level 3, has resulted in increase in enzyme production. Increase in pH has resulted in higher polygalacturonase expression up to level 2 (pH 4.5) and subsequent increase resulted in decrease in the polygalacturonase yield. While in case of incubation time, the polygalacturonase yield was higher up to level 3; subsequent increase in the incubation time (level 4) reduced the yield. Also, it is evident from optimum condition results that the PG yield can be increased from 102 to 115.5 U mL^{-1} i.e., overall 13% enhancement in the enzyme production.

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