Brazilian Journal of Microbiology (2011) 42: 794-806
ISSN 1517-8382

SCREENING AND OPTIMIZATION OF PECTIN LYASE AND POLYGALACTURONASE ACTIVITY FROM GINSENG PATHOGEN *CYLINDROCARPON DESTRUCTANS*

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Submitted: August 30, 2009; Returned to authors for corrections: February 11, 2010; Approved: January 13, 2011.

ABSTRACT

Cylindrocarpon destructans isolated from ginseng field was found to produce pectinolytic enzymes. A Taguchi's orthogonal array experimental design was applied to optimize the preliminary production of polygalacturonase (PG) and pectin lyase (PL) using submerged culture condition. This method was applied to evaluate the significant parameters for the production of enzymes. The process variables were pH, pectin concentration, incubation time and temperature. Optimization of process parameters resulted in high levels of enzyme (PG and PL) production after ten days of incubation at a pH of 5.0 at 25°C in the presence of 1.5% pectin. Among different nitrogen sources, urea and peptone showed high production of PG and PL, respectively. The enzyme production and mycelial growth seems to have direct influence on the culture conditions; therefore, at stationary state high enzyme production and mycelial growth were obtained than agitation state. Along with this, optimization of enzyme activity was also determined using various physiological parameters like, temperature, incubation time and pH. Taguchi's data was also analyzed using one step ANOVA statistical method.

Key words: *C. destructans*; polygalacturonase; pectin lyases; ginseng

INTRODUCTION

In plant cells, pectin is a family of complex polysaccharides that contain α-1, 4-linked D-galacturonic acid. Pectic substances constitute the main components of the middle lamella that is, the intercellular cement between plant cells where it helps to bind cells together. It also makes up a large portion of a primary cell wall, in which they form an amorphous gel, filling the spaces between the cellulose microfibrils. And also play a major role in cell wall extension and fruit softening (11). Pectic substances are polysaccharides consisting of mostly the chains of galacturonan molecules interspersed with a much smaller number of rhamnose molecules and side chains of galacturonan and some other five carbon sugars. The enzymes that degrade pectic substances are known as pectinases or pectinolytic enzymes. Some chain-

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splitting pectinases, called polygalacturonases (PG: EC 3.2.1.15), split the pectin chain by adding a molecule of water and breaking (hydrolyzing) the linkage between two galacturonan molecules; pectin lyases (PL: EC 4.2.2.2), split the chain by removing a molecule of water from the linkage, thereby breaking it and releasing products with an unsaturated double bond. Both the enzymes break the pectin chain at random sites or terminal linkage (exo-pectinases) of the chain and release single unit of galacturonan.

The pectin degrading enzymes have been shown to be involved in the production of many diseases, particularly those characterized by soft-rotting of tissues. Pectin degradation results in liquefaction of the pectic substances that hold plant cells together and in weakening of the cell walls and it leads to tissue maceration. Many phytopathogens produce these enzymes to invade into the plants, including *Cylindrocarpon destructans,* produce drastic root rot diseases in plants, especially in ginseng. The infection in roots can result in complete decay; hence, the name "disappearing root rot" frequently used to describe this disease (19). Previous research has shown that the fungus *Cylindrocarpon destructans* (teleomorph *Nectria radicicola*) is a major root rot pathogen of ginseng (18, 20, 21).

Ginseng is an important cash crop in various regions of China, Korea, North America and other parts of countries. The ginseng roots are taken orally as adaptogens, aphrodisiacs, nourishing stimulants, and in the treatment of type II diabetes. In recent study, the ginseng proved to have anti-inflammatory effect, as well as it reduces fatigue in cancer patients (13). Hence, serious root rot disease in ginseng caused by *C. destructans* can result in yield losses of up to 25-30% (7, 23).

In this study, the production of pectinolytic enzymes was optimized to determine the severity of pathogenesis. For optimization, various parameters are chosen which will have direct influence on the enzyme production therefore; temperature, pH, incubation period, and substrate concentrations were applied using Taguchi's method of orthogonal array (25). In this array, the columns are mutually orthogonal. That is, for any pair of columns, all combinations of factor levels occur; and they take place an equal number of times.

The preliminary study using Taguchi's method served for the determination of initial levels of the production since no literature information was available on the production from the fungus *C. destructans* and different nitrogen source, sodium chloride concentrations were studied along with this comparative analysis of enzyme production by stationary condition versus agitation condition was also determined.

After uttering PG and PL production, their activities were optimized using various physiological factors, which will escort a complete lesson of the particular enzyme from *C. destructans*.

MATERIALS AND METHOD

Source of organism and inoculants' preparation

The fungus used for this study *C. destructans* (KACC 41077) was purchased from KACC (Korean Agriculture of Culture Collection, South Korea) which was isolated from the soil of the ginseng field. The propagation of the culture was maintained on the PDA (Difco) plates. The fungal strain cultured on PDA plates for seven days at 25ºC was then washed with sterile water to extricate spores. The resulting spore suspension was used as inoculants for the experiments.

Taguchi's orthogonal array

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. 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. The symbolic designation of these arrays indicates the main information on the size of the experimentation, L16, has 16 trails. The total degree of freedom available in an orthogonal array is equal to the number of trails minus one. In this study four columns are designed with different factors as shown in Table 1. Each of these factors assigned with four levels, therefore, 1, 2, 3 and 4 levels respectively. Table 2 shows the experimental condition designed using L16 orthogonal array system. All the parameters were conducted using Czapek Dox mineral medium supplemented with apple pectin (Sigma, South Korea) containing 1 g KH_2PO_4, 2 g $NaNO_3$, 0.50 g $MgSO_4.7H_2O$, 0.05 g KCl, 0.01 g $FeSO_4$, in 1 L.dH_2O and incubated at appropriate temperatures without agitation. Based on the experimental days the liquid cultures were filtered through Whatman No.1 filter paper and centrifuged at 5000 *g* for 20 min. The protein concentration was assayed using Bradford's method. The supernatant was stored at -20ºC.

Table 1. Taguchi's experimental design matrix and production of pectinolytic enzymes by *C. destructans*

	Levels			
Trail No.	**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. Variables (factors) and their levels used in Taguchi's method

Factors	Levels			
	1	2	3	4
pH	4.5	5.0	6.0	7.0
Incubation time (days)	2.0	6.0	8.0	10.0
Pectin concentration (%)	7.5	1.0	1.3	1.8
Temperature (ºC)	23.0	25.0	28.0	30.0

Analysis of Taguchi's experiment

Once the experimental design has been determined and the trails have been carried out, the measured performance characteristic from each trail can be used to analyze the relative effect of the different parameters. To determine the effect of each variable has on the output, the signal-to-noise ratio, or the SN number, needs to be calculated for each experiment conducted. The calculation of the SN for the first experiment in the array above is shown below in the equations (1) below,

$$S/N\ (dB) = -\ 10 \log \sum_{i=1}^{n} y_i^2 / n = -\ 10 \log (y^2 + S^2) \quad (1)$$

After calculating the SN ratio for each experiment, the average SN value is calculated for each factors and level.

Once these SN ratio values are calculated for each factor and the range R (R = high SN - low SN) of the SN for each parameter is calculated and entered. The larger the R values for a parameter, the larger the effect of the variable as on the process. This is because the same change in signal causes a larger effect on the output variable being measured.

The effect of this factor is then calculated (2) by determining the range:

$$\Delta = Maximum - Minimum \quad (2)$$

Apparently deposition rate has the largest effect on the processor yields and that temperature has the smallest effect on the processor yield.

Optimization of enzyme production using nitrogen source / NaCl concentration / Agitation and stationary culture condition

The enzyme productions were further optimized using new parameters designed by captivating the best trail parameter from the Taguchi's method. The effect of nitrogen sources and sodium chloride concentrations were checked to determine the best nitrogen source and influence of sodium chloride in the production of enzyme. The fungus was grown on the different nitrogen sources such as, urea, yeast extract, beef extract, peptone, KNO_3 and $NaNO_3$ at the concentration of 0.5% level. The consequence of sodium chloride on enzyme production by *C. destructans* was carried out by incorporating NaCl at a range of 0-2.5% into the culture medium. Besides with the enzyme production, mycelial dry weight was also determined. The culture broth was filtered through the pre-weight Whatman No.1 filter paper, followed by drying to constant weight at 40ºC for about 24 h. The final biomass was expressed as dry cell weight (g/ml). Growth rate (biomass) of fungus was also estimated between the agitated culture (180 rpm at 25ºC) and the stationary culture (incubated at 25ºC) using the standard mycelia dry-weight method, in addition to, enzyme activity was also checked.

Optimization of enzyme activity

The polygalacturonase and pectin lyase activities were carried out using different parameters as follow:

Incubation time: The effect of time on enzyme activity was determined by incubating the reaction mixture up to the appropriate times, i.e., 10-120 min.

pH: The impact of pH on enzyme activity was studied by adjusting the pH to acquire desired ranges from 3.5-8.5 using 1 N NaOH and 1N acetic acid.

Temperature: The enzyme reaction was carried out in different temperatures at 25, 30, 37, 40, and 70ºC, the maximum activity was checked.

Protein determination

Protein content of the culture filtrates were determined by Bradford's method using BioRad reagent and bovine serum albumin (BSA) as standard (6).

Enzyme assay

Polygalacturonase activity was checked by quantifying end groups released during the reaction and it was assayed using the procedure of Panda *et al*. (15). The amount of substrate (polygalacturonic acid) and enzymes were 0.4 and 0.086 ml dissolved in 0.05 M sodium acetate buffer (pH 5.2), respectively. The reaction carried out by incubating at 37ºC. One unit of enzyme activity was the amount of enzyme catalyses the release of 1 µmol of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions. Galacturonic acid (Sigma) was used as standard for calibration of enzyme activity. The calculated eq. (3)

$$Activity\left(\frac{U}{ml}\right) = \left(\frac{mg\ of\ galacturonic\ acid}{212.12}\right)\left(\frac{1}{20}\right)\left(\frac{1}{0.086}\right) \quad (3)$$

Pectin lyase activity was determined spectrophotometrically by the procedure described in Alana *et al*. and Albersheim (1, 3). The reaction mixture (1.25 ml) contained 0.1 mol/l citrate phosphate buffer (pH 6.0), 0.5% (w/v) citrus pectin (D.E.), and the appropriate volume of crude enzyme extract (final concentration 1 pg of protein per ml). This mixture, without substrate, was pre-incubated at 40°C for 15 min and the reaction initiated with the addition of the substrate and again incubated for 60 min. Enzyme inactivated by boiling for 3 min was used as a control. One unit of activity is the amount of enzyme which produces an increase of 1 unit of A_{235}, per min.

RESULT

Taguchi's experiment result analysis

Cylindrocarpon destructans are phytopathogenic fungus causing rot diseases in plants especially rusty rot in ginseng, which leads to yield loss. Submerged fermentation experiments

study with the designed experimental condition showed significant variation in the polygalacturonase activity and pectin lyase activity from *C. destructans* (Fig. 1). Production levels were found to be highly dependent on the culture conditions. An L16 orthogonal experimental design was used to investigate four different culture components such as, pH, incubation time, amount of pectin as carbon source/inducer and temperature. 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 and pectin lyase productions were pH 5, 10 days of incubation and 1.5% pectin and incubation temperature at 25°C (Trial 8). The average effect of the factors at the assigned levels on the polygalacturonase production by *C. destructans* was shown in Table 3. This table shows the influence of four individual factors (pH, incubation time, pectin and temperature) on the polygalacturonase and pectin lyase yields. Individually at level stage, pH has the highest effect in level 4 with 32.012 U ml^{-1} and whereas pectin as carbon source/inducer, temperature and incubation time show their higher effects in level 3 with 29.7 U ml^{-1}, level 4 with 27.31 U ml^{-1} and level 4 with 29.7 U ml^{-1}, respectively on the polygalacturonase yield (Table 3). And pectin showed highest effect in level 3 with 30.4 U ml^{-1} for PL, and whereas incubation time, temperature, and pH show their higher effects in level 3 with 29.81 U ml^{-1}, level 3 with 26.41 U ml^{-1} and level 2 with 28.16 U ml^{-1}, respectively on the pectin lyase yield. Culture pH and pectin concentration is one of the important parameter in fungal cultivation.

The signal –noise ratio were determined to obtain R-value for ranking the factors based on their contribution on the enzyme production. Fig. 2 shows the R- value of each factor that contributes in the enzymes production. In order to conduct an analysis of the relative importance of each factor more systematically, an analysis of variance (ANOVA) was applied to the data. The main objective of ANOVA is to extract from the results how much variations each factor causes relative to the total variation observed in the result. From the results of ANOVA in Table 3a, the pH and pectin concentration factors had the largest variance for PG and PL, respectively. The variance within the factors and between the factors was analyzed (Table. 3b).

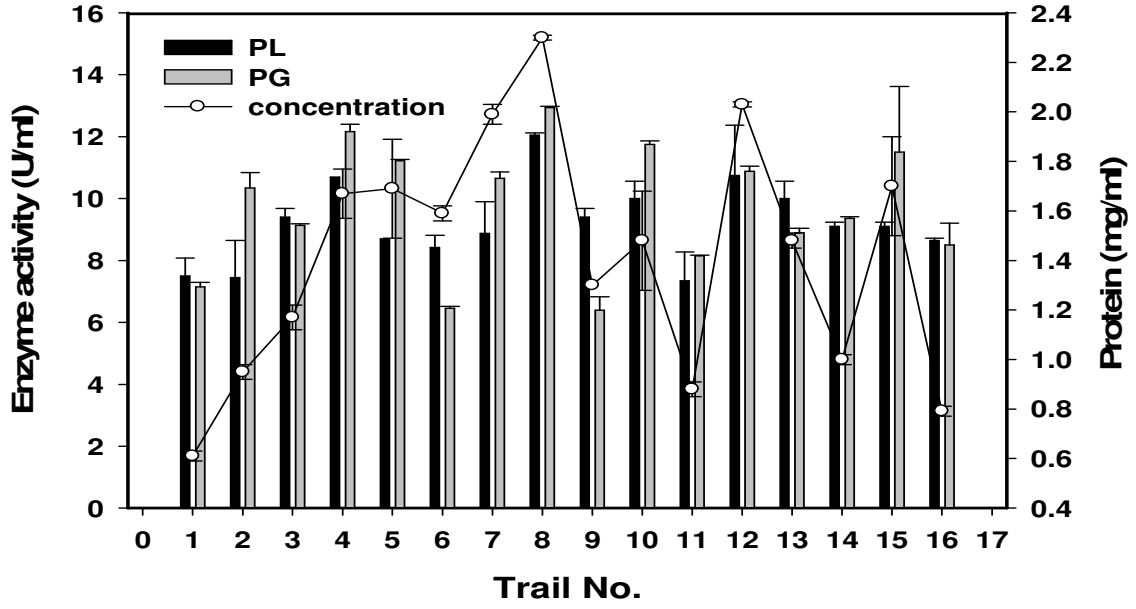

Figure 1. Polygalacturonase and pectin lyase production by *C. destructans* in a submerged culture using Taguchi orthogonal array (Three replicates).

Table 3a. Analysis of Variance (ANOVA)

(i) Polygalacturonase

Factors	df	Sum	Mean	Sum sq	SS	Variance	St.dev.
pH	3	98.30	24.56	2491.01	75.15	25.05	5.00
Incubation time	3	101.20	25.30	2593.50	33.14	11.05	3.32
pectin	3	98.35	24.59	2459.37	41.18	13.72	3.70
Temperature	3	97.21	24.30	2395.58	33.14	11.05	3.32
Errors	34						
Total	46	395.10	24.69	9939.47	184.79	12.32	3.50

(ii) Pectin lyase

Factors	df	Sum	Mean	Sum sq	SS	Variance	St.dev.
pH	3	105.44	26.36	2799.89	20.49	6.83	2.61
Incubation time	3	105.44	26.36	2827.55	48.15	16.00	4.00
pectin	3	105.43	26.35	2850.77	71.90	23.96	4.90
Temperature	3	103.66	25.915	2697.47	11.12	3.70	1.90
Errors	34						
Total	46	419.97	26.25	11175.67	152.25	10.15	3.20

[a]Variances and standard deviations are calculated with denominator = n-1.

Table 3b. Summary of ANOVA

(i) Polygalacturonase

Source	SS	df	MS	F-ratio	P-value
Between groups	2.18	3	0.73	0.05	0.98
within groups(error)	182.61	12	15.22		
Total	184.79	15			

(ii) Pectin lyase

Source	SS	df	MS	F-ratio	P-value
Between groups	0.59	3	0.20	0.02	0.99
within groups(error)	151.66	12	12.64		
Total	152.25	15			

*SS, Sums of square; df, degree of freedom; MS, mean squares.

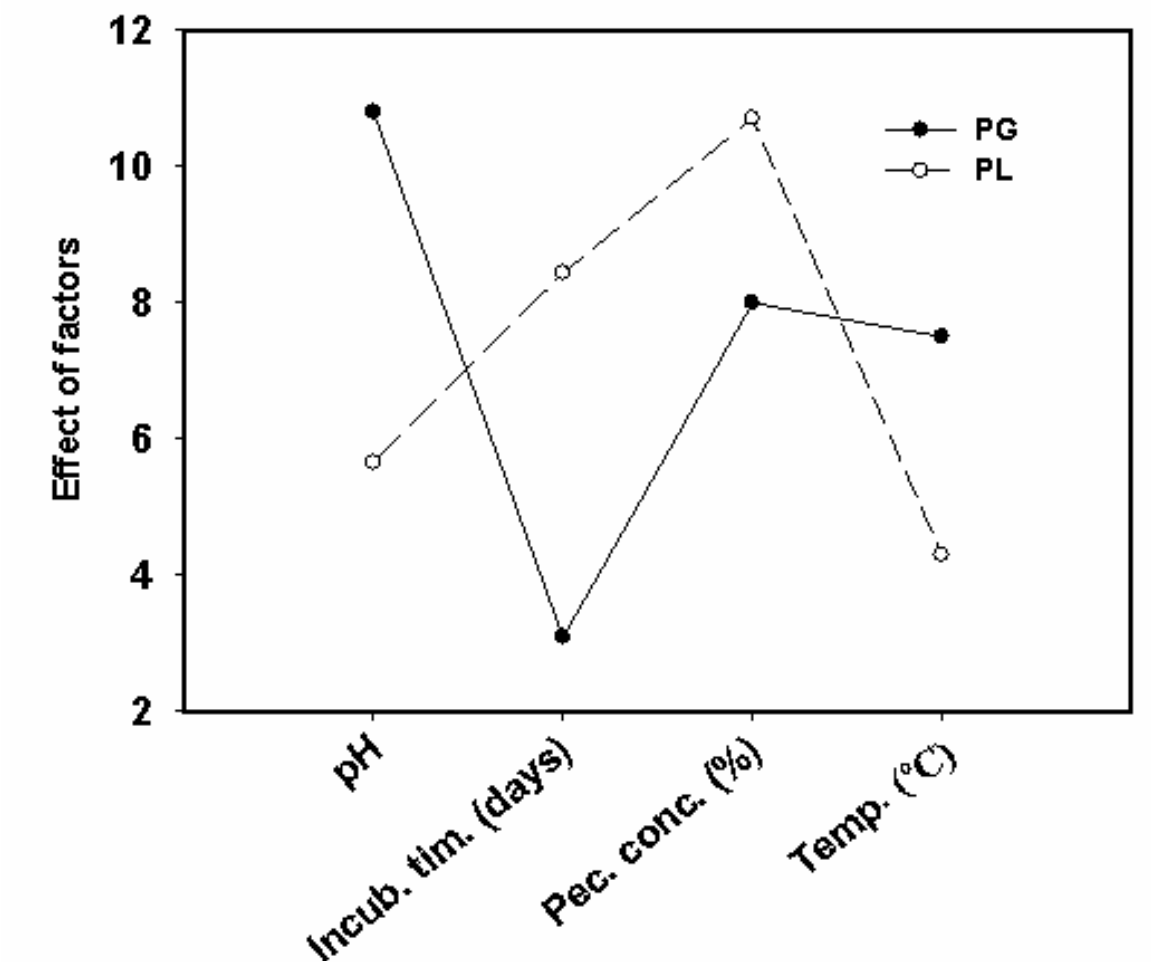

Figure 2. The graphs shows the effect of each factor contribution in the enzyme production.

Effect of nitrogen source / NaCl concentration / Agitation and stationary culture condition

Among the various nitrogen source used, urea and peptone gave maximum polygalacturonase activity (12.0 U ml^{-1}) and pectin lyase activity (11.7 U ml^{-1}), from the results, nitrogen source also constitute a profound effect on pectinolytic activity on culture filtrate. Whereas, the biomass weighed high on yeast extract with 3.86 g dry weight, hence this result confirmed that the yeast extract was only used in the growth, not on the enzyme production (Fig. 3). Sodium chloride was not found to exert a significant role on enzyme production. Even in its absence, considerable activities were found. Fig. 4 shows the NaCl effect, in which 0.5% showed maximum biomass of 3.3 g (dry weight) as well as paramount PG (13.5 U ml^{-1}) and PL (12.5 U ml^{-1}) activities. Thereafter, increase in the salt concentration resulted in drastic reduction in enzyme production. The effect of stationary culture influenced the production of both PG (12.0 U ml^{-1}) and PL (11.5 U ml^{-1}), thus the biomass weighed 3.31 g (dry weight), and this shows the stationary culture influence the production of pectinolytic enzymes than agitation. Agitation had an inhibitory effect on pectic enzyme production as shown in Table 4. This could be as a result of catabolite repression by other substances that are enhanced by agitation in the cultures.

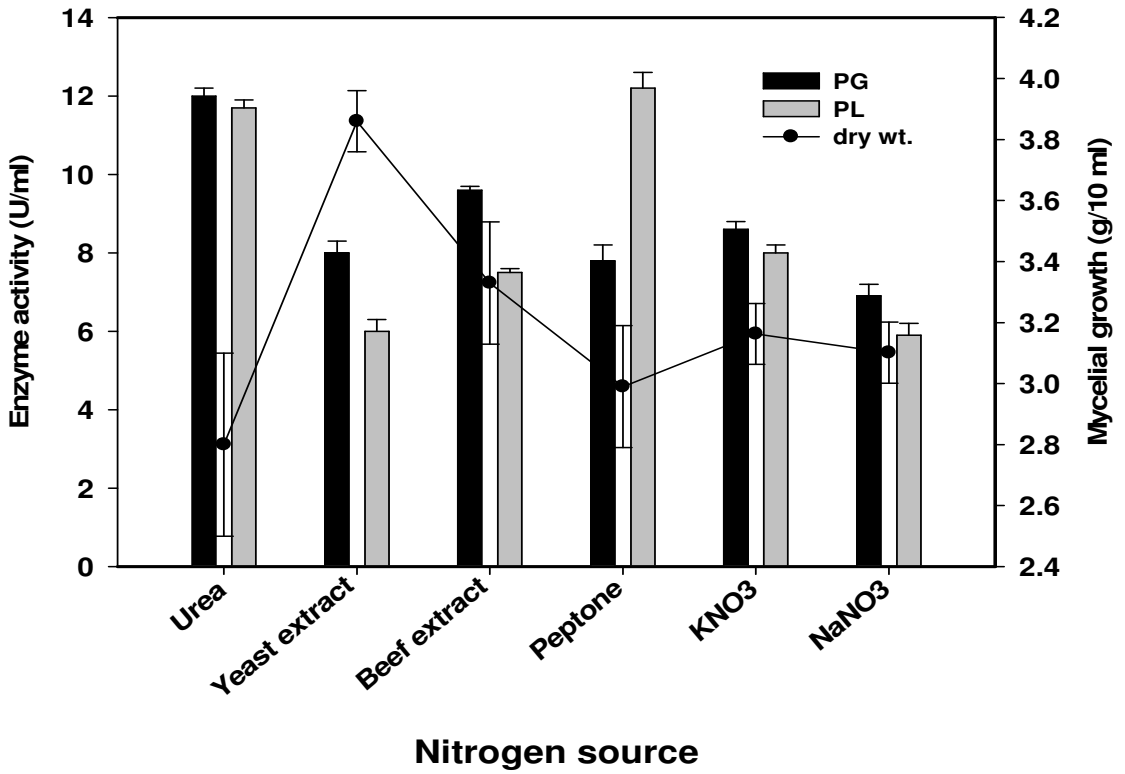

Figure 3. Effect of various nitrogen sources (0.5%) on enzyme production by *C. destructans* grown in culture medium (pH 5.0) containing 1.5% pectin at 25°C.

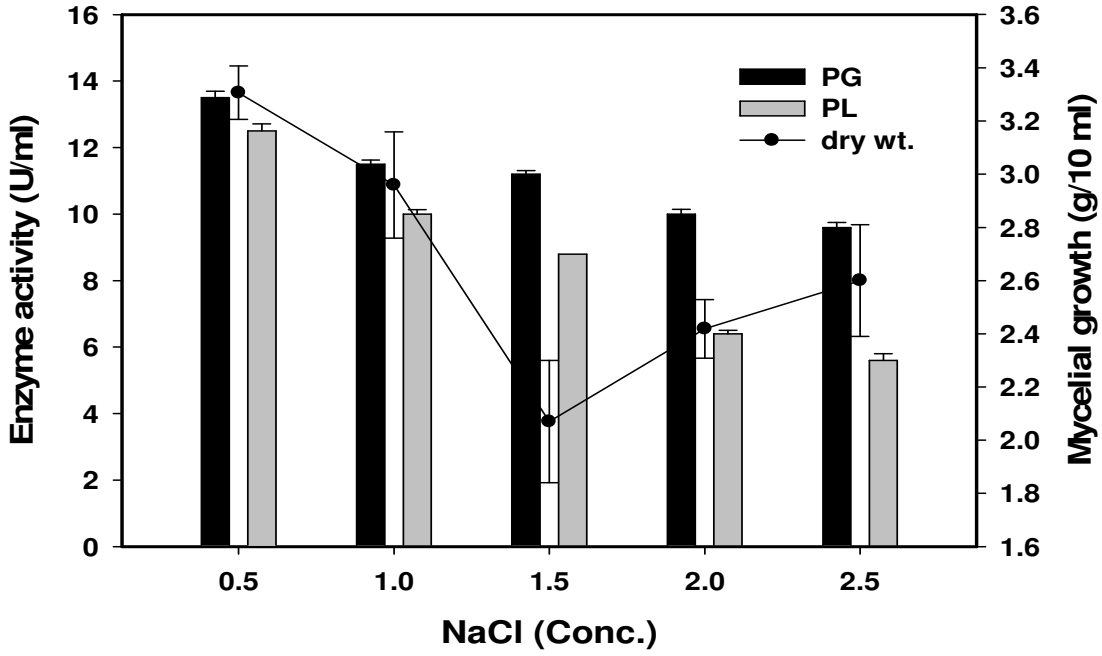

Figure 4. Effect of sodium chloride concentration on enzyme production by *C. destructans* grown in culture medium (pH 5.0) containing 1.5 % pectin at 25ºC.

Table 4. The effect of agitation and stationary culture technique on growth, PG and PL activity by *C. destructans* cultured for 10 days

	Agitation at 25ºC	Stationary at 25ºC
Mycelial dry weight (g 10 ml^{-1})	0.67	3.31
Protein content (mg ml^{-1})	0.78	1.13
Polygalacturonase activity (U ml^{-1})	8.80	12.00
Pectin lyase activity (U ml^{-1})	6.00	11.50

Optimum incubation time, pH and temperature for pectinolytic activity

The 0.1 U of PG and PL were used to check optimum activity. The incubation time is one of main factor that interprets the enzyme activity; we observed both PG and PL activities elevated at the incubation time of 60 min and decreased gradually (Fig.5). The optimum pH for the catalytic reaction was determined from the results shown in Fig. 6. The polygalacturonase and pectin lyase are most begun at pH 4.5 and 5.5 and completely inactivated at pH values above 6.5 or below 3.2 (result not shown). The result in Fig.7 shows the maximum activity at the temperature of 37 and 30ºC for polygalacturonase and pectin lyase respectively. Thus it showed the mesophilic nature of the fungi.

The overall result shows maximum pectin lyase and polygalacturonase activity at 30ºC for 60 min with the pH 5.5 and 37ºC for 60 min with pH 4.5, respectively.

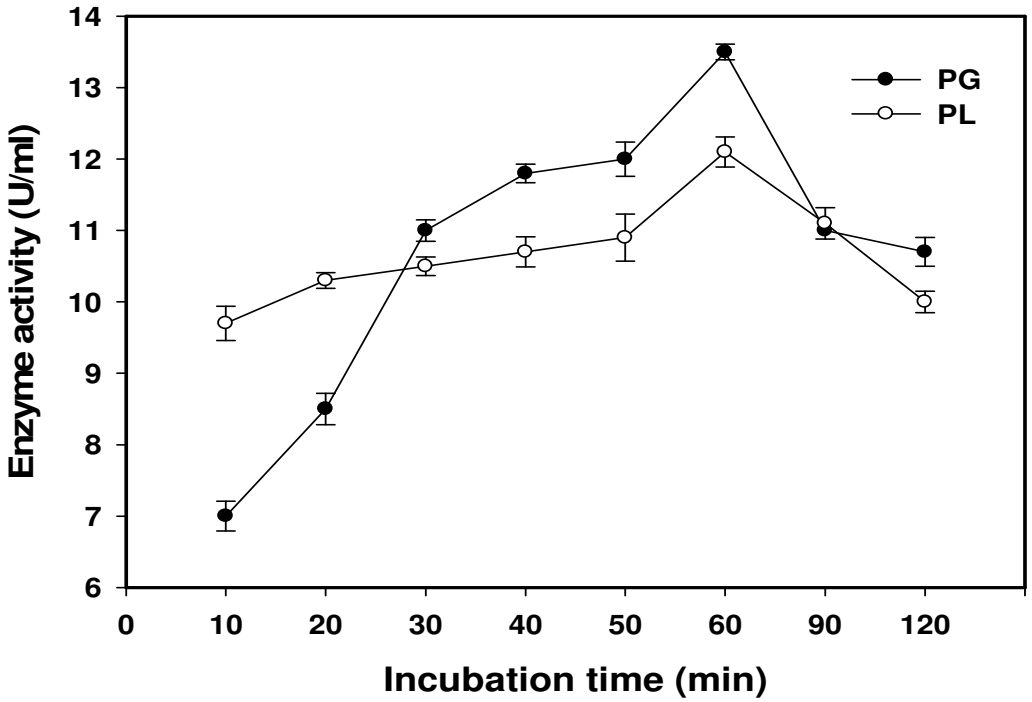

Figure 5. Determining of incubation time for optimum enzyme activity.

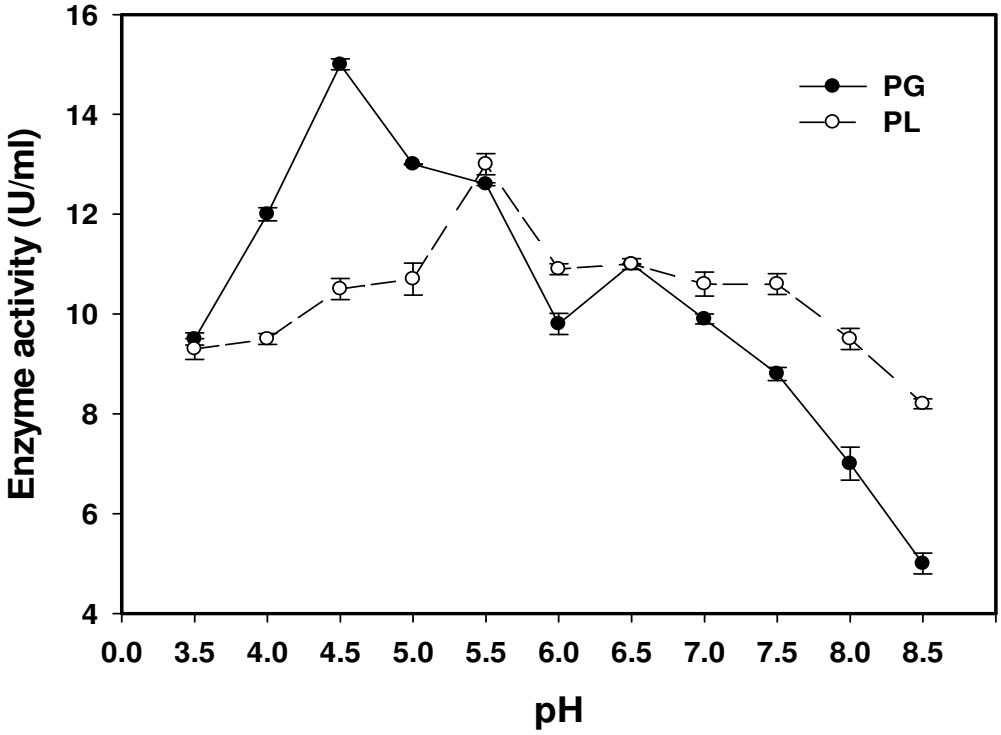

Figure 6. Effect of pH on enzyme activity of polygalacturonase and pectin lyase.

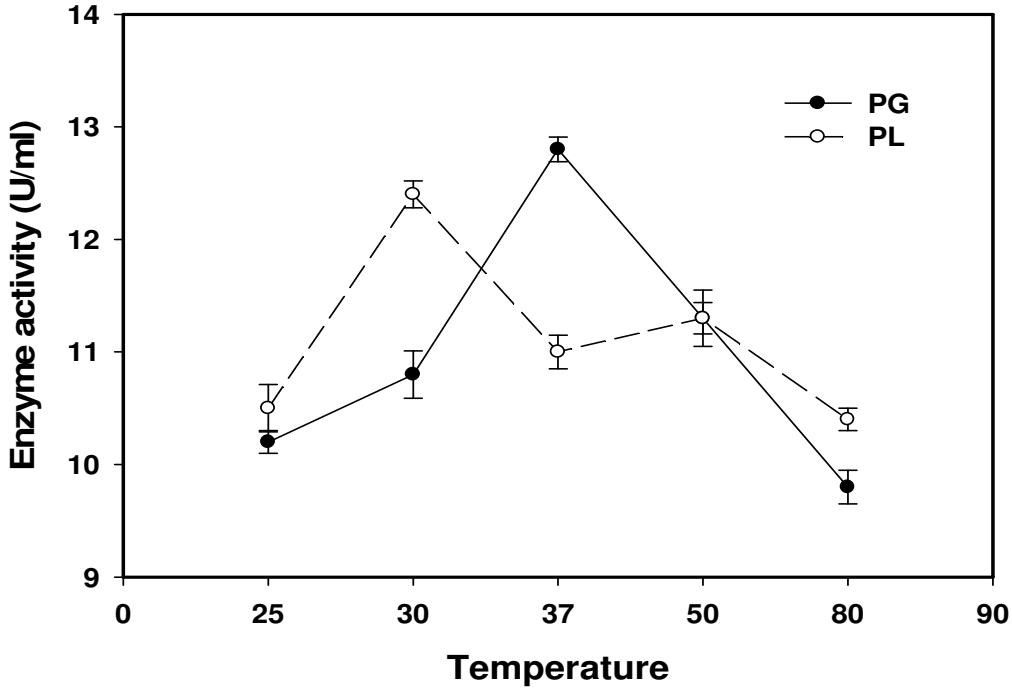

Figure 7. Effect of temperature (°C) on enzyme activity.

DISCUSSION

To date, no clear reports are available in literature regarding the optimization conditions for PG and PL enzyme production by *C. destructans*, so we studied preliminary production of these enzymes. From our research, we determined the optimum conditions for the production of pectinolytic enzymes (PG and PL) from *C. destructans* using Taguchi's orthogonal array. The optimum conditions for the production of PG and PL was detected in ten days at temperature 25 with 1.5% pectin in acidic pH of 5.0. Based on the Taguchi's data calculation, pH shows more contribution in the production of PG, where as pectin concentration for PL. The Taguchi method reduced research and development costs by improving the efficiency of generating information needed to design systems that are insensitive to usage conditions, manufacturing variation, and deterioration of parts. As a result, development time can be shortened significantly; and important design parameters affecting operation, performance, and cost can be identified. Furthermore, the optimum choice of parameters can result in wider tolerances so that low cost components and production processes can be used. Thus, manufacturing and operations costs can also be greatly reduced.

The nitrogen sources also have an important part in the production of enzymes where urea and peptone showed increased activity of PG and PL, similar data have been reported in *Penicillium* strain (14) where the non-defined organic urea was found better than the defined nitrogen sources for maximal PG production. NaCl content neither enhanced nor destroyed the production of enzyme; our investigation also supports the data proved by Mathew *et al*. (14) in *Penicillium* sp. It is interesting that although *C. destructans* is not a halophile, still the enzymes were stable in high salt concentrations. The pectinolytic enzyme production was favored by stationary culture condition rather than agitated condition. Dronawat *et al*. (8) reported an increase in gluconic acid concentration in cultures of *A. niger* with increasing agitation, it could be a reason of less enzyme production.

Optimum pH for enzyme (PG and PL) activity was observed in the acidic pH; our result gave good agreement with reported data in which, the pH between 4.0-5.0 was reported to give maximum activity of pectinolytic enzymes in *Penicillium italicum* (2), *Aspergillus niger* (9), *Fusarium oxysporum* and *Fusarium equiseti* (5). Optimum production of pectic enzymes from many moulds has been reported to be within the acidic pH range (24, 26). The incubation period of 60 min of reaction time increased the PG and PL activity, at mesophilic (37 and 30) temperature, which proved to be the nature of these enzymes. The same thermolabile nature was also reported in *Aspergillus fumigatus* by Phutela *et al*. (17). Many fungal PGs are thermolabile and become irreversibly inactivated by 60°C with a few exceptions such as *Penicillium* (10), *Rhizopus* (22), and *Sclerotinia* (4).

Taguchi's approach to guideline design provides the systematic and efficient method for determining near optimum design parameters for performance and cost (12, 16, 25). Taguchi experimental design involves 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, establishing the relationship among variables and also the performance at the optimum levels. By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process can be predicted and 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). 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). Furthermore, the conclusions drawn from small scale experiments are valid over the entire experimental region spanned by the control factors and their settings. Therefore, this research will serve as a base line, and it will provide us to work further in detailed study of PG and PL production in this fungus. The purification and the characterization of polygalacturonase from *Cylindrocarpon destructans* are in process.

ACKNOWLEDGEMENT

This study was supported by grant from the KGCMVP for Technology Development Program of Agriculture and Forestry, Ministry of Food, Agriculture, Forestry and Fisheries, Republic of Korea.

REFERENCE

1. Alana, A.; Alkorta, I.; Dominguez, J.B.; Liama, M.J.; Serra J.L. (1990). Pectin lyase activity in a *Penicillium italicum* strain. *Appl. Environ. Microbio*. 56, 3755-3759.
2. Alana, A.; Gabilondo, A.; Hernando, F.; Moragues, M.D.; Dominquez, J.B.; Liama, M.J.; Sessa, J.L. (1989). Pectin lyase production by a *Penicillium italicum* strain. *Appl. Environ. Microbiol*. 55, 1612-1616.
3. Albersheim, P. (1966). Pectin lyase from fungi. *Methods* 2 *Enzymol.* 8, 628-631.
4. Archer, S.W.; Fielding, A. (1975). Thermostable polygalacturonase secreted by *Sclerotinia fructigana. J. Food Sci.* 40, 423-424.
5. Bahkali, A.H.; AI-Khaliel, A.H.; Elkhider, K.A. (1997). In vitro and In vivo production of pectinolytic enzymes by some phytopathogenic fungi isolated from Southwest Saudi Arabia. *Sci.* 2, 125-137.
6. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72, 248-54.
7. Chung, H.S. (1975). Studies on *Cylindrocarpon destructans* (Zins.) Scholten causing root rot of ginseng. *Rep. Tottori. Mycol. Inst*. 12, 127-38.
8. Dronawat, S.N.; Svihla, C.K.; Hanley, T.R. (1995) The effects of agitation and aeration on the production of gluconic acid by *Aspergillus niger*. *Appl. Biochem. Biotechnol*. 51/152, 347-354.
9. Fawolea, O.B.; Odunfab, S.A. (2003). Some factors an ecting production of pectic enzymes by *Aspergillus niger*. *Int. Biodeterior. Biodegrad.* 52, 223 -227.
10. Gillespie, A. M.; Cook, K.; Coughlan, M. P. (1990). Characterization ofendopolygalacturonase produced by solid-state cultures of the aerobic fungus *Penicilliuni capsulatum. J. Biotechnol.* 13, 279-292.
11. Jacob, N.; Asha Poorna C.; Prema P. (2008). Purification and partial characterization of polygalacturonase from *Streptomyces lydicus*. *Bioresource Technol*. 99, 6697-670.
12. Kackar, R.N. (1985). Off-line quality control, parameter design and Taguchi methods, *J. Qual Technol*. 17, 176-88.
13. Kook, S.; Han, H.K; Kim, G.H.; Choi, K. (2008). The anti-hepatotoxic effect of ginseng in rats: meta-analysis. *J. Ginseng Res*. 29, 161-170.
14. Mathew, A.; Abi N.E.; Molly, A.G. (2008). Optimization of culture conditions for the production of thermostable polygalacturonase by *Penicillium* SPC-F 20. *J. Ind. Microbiol. Biotechnol*. 35, 1001-1005.
15. Panda, T.; Naidu, G.S.N.; Sinha, J. (1999). Multiresponse analysis of microbiological parameters affecting the production of pectolytic enzymes by *Aspergillus niger*: a statistical view. *Process Biochem*., 35, 187-95.
16. Phadke, S.M. (1991). Quality Engineering Using Robust Design, Prentice Hall, Englewood Cliffs, N.J., Presented at the Annual Conference of International Society of Parametric Analysts.
17. Phutela, U.; Dhuna, V.; Sandhu, S.; Chadha, B.S. (2005). Pectinase and polygalacturonase production by a thermophilic *Aspergillus fumigatus* isolated from decomposting organge peels. *Braz. J. Microbiol*. 36, 63-69.
18. Punja, Z.K. (1997). Fungal pathogens of American ginseng (*Panax quinquefolius* L.) in British Columbia, Canada. *Can. J. Plant Pathol*. 19, 301-306.
19. Rahman, M.; Punja, Z.K. (2005). Factors influencing development of root rot on ginseng caused by *Cylindrocarpon destructans*. *Phytopathol*. 95, 1381-1390.
20. Reeleder, R.D.; Brammal, R.A. (1994). Pathogenicity of *Pythium* species, *Cylindrocarpon destructans*, and *Rhizoctonia solani* to ginseng seedlings in Ontario. *Can. J. Plant Pathol*. 16, 311-316.
21. Reeleder, R.D.; Roy, R.; Capell, B.B. (1999). Seed and root rots of ginseng (*Panax quinquefolius)* caused by *Cylindrocarpon destructans* and *Fusarium* spp. *J. Ginseng Res*. 26, 151-158.
22. Ros, J.M.; Saura, D.; Saimeron, M.C.; Lencina, J. (1993). On the response under different treatments of the thermostable endopolygalacturonase produced by *Rhizopus nigricans. Z. Lebensm.-Unters. Forsch.* 1965, 356-359.
23. Seifert, K.A.; McMullen, C.R.; Yee, D; Reeleder, R.D; Dobinson, K.F. (2003). Molecular differentiation and detection of ginseng adapted isolates of the root rot fungus *Cylindrocarpon destructans*. *Phytopathol.* 93, 1533-42.
24. Shin, I.; Dowmez, S.; Kilic, O. (1983). Study on pectolytic enzyme

production from some agricultural wastes by fungi. *Chemie Microbiologie Technologie der Lebensm.,* 8, 87-90.

25. Taguchi, G. (1991). Introduction to Quality Engineering, Asian Productivity Organization (Distributed by American Supplier Institute Inc., Dearborn, MI). Presented at the Annual Conference of International Society of Parametric Analysts.
26. Zetelaki-Horvath, K. (1980). Factors affecting pectinase activity. *Acta Aliment*. 10, 371-378.