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Utilization of *Aspergillus oryzae* to produce pectin lyase from various agro-industrial residues

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ABSTRACT

The present study was aimed to investigate the culture influence on pectin lyase production potential of fungal strain *Aspergillus oryzae*. The enzyme profile of *A. oryzae* showed highest activity of pectin lyase after 3rd day of incubation on lemon peel waste under solid state fermentation conditions. To induce the pectin lyase synthesis capability of *A. oryzae* at optimal level various culture variables including physical and nutritional parameters were optimized by adopting classical optimization technique. Therefore, through fermentation process optimization the production of pectin lyase was substantially induced up to the level of 875 U/mL, when fermentation medium of lemon peel waste inoculated with 5 mL spore suspension of *A. oryzae*. The optimal fermentation conditions for maximum pectin lyase yield were as: optimum pH 5, 70% moisture level and incubated at 40 °C in addition with 1% sterile glucose solution as readily available carbon source and 0.2% yeast extract as an inexpensive nitrogen supplement (1%). The results obtained in current investigation so far demonstrated that culture conditions have great influence on the pectin lyase production potential of *A. oryzae*.

1. Introduction

Pectin containing substances are among the most value-added materials from the citrus family which can acts as inducer for the production of industrially relevant enzymes particularly pectin lyase by means of biological processing of such waste materials (Irshad et al., 2014). Throughout the world a large magnitude of various agricultural and agro based

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industrial waste residues are being generated from current industrial processing practices. A wide range of agricultural/agro-industrial wastes and by-product residues such as sugar cane bagasse, oranges, rice, banana and coffee are potentially suitable feed-stocks for their possible bio-conversion into a range of value-added product of interests like enzymes, chemicals and many other fine materials (Anwar, Gulfraz, & Irshad, 2014; Giese, Dekker, & Barbosa, 2008; Ibrahim, El-Zawawy, Jüttke, Koschella, & Heinze, 2013; Iqbal, Kyazze, & Keshavarz, 2013). Orange peel contains large amounts of soluble carbohydrates, particularly fructose, glucose, sucrose, and pectin, which can be used as a resourceful ingredients to obtain wide range of quality products (Liu, Fishman, & Hicks, 2007; Rosales, Couto, & Sanromán, 2002).

Solid state fermentation (SSF) is a microbial involved process which takes place under minute moist conditions or in the complete absence of free flowing water contents in the growth/fermentation media (Iqbal, Asgher, & Bhatti, 2011). Enzyme production in solid-state fermentation is higher than in submerged fermentation which is mainly because, there are several advantages of SSF, for example high productivities, extended stability of products and low production costs, this makes SSF the preferred technique for special fields of application such as the production of enzymes and food. With increasing progress and application of rational methods in engineering, SSF will achieve higher levels in standardization and reproducibility in the future (Iqbal et al., 2011, 2013; Rosales et al., 2002).

By keeping in mind the extensive industrial and biotechnological applications of pectin lyase the present study reports on the influence of culture conditions on pectin lyase production potential of fungal strain *Aspergillus oryzae* under SSF. In the present study, a novel approach was applied that dealt entirely with the use of different residual wastes from various field crops (waste materials from wheat straw, apple pomace, lemon peel, orange peel and municipal paper) to obtain the product of industrial interest. The present study was also focused on providing a potential solution for the management of a large magnitude of solid wastes.

2. Materials and methods

2.1. Chemicals, reagents and agro-industrial wastes

All the chemicals and reagents used were of analytical grade. The agro based waste materials, *i.e.,* wheat straw waste, apple pomace waste, lemon peel waste, orange peel waste, and municipal paper waste were collected from a local fruit market and the routinely binned local newspapers respectively. All of the collected materials were crushed, washed several times with hot water, dried (at 60 °C), then ground into a suitable mesh particle size of 40 mm and finally stored in plastic jars and used for further subsequent experiments.

2.2. Fermentative organism and culture condition

The pure fungal strain of *A. oryzae* available in the Department of Biochemistry; University of Gujrat, Pakistan was grown on potato dextrose agar (PDA) slants and used as fermentation organism. The fully grown culture was stored at 4 °C and the preserved culture was re-cultured periodically after every 15 days on fresh PDA slants to maintain the viability of the strain.

2.3. Fungal spore suspension development

The composition of medium used for the production of pectin lyase is as follow (g/L): glucose 2; $MgSO_4 \cdot 7H_2O$ 0.05; $CaCl_2 \cdot 2H_2O$, 0.1; NH_4Cl, 0.12 and thiamine, 0.001 and pH 6.0 with silica gel chips (1.2 mm, dia) in conical flask plugged with cotton wool and sterilized at 15 lbs/in^2 pressure (121 °C) for 15 min. Two milliliter of the spores was transferred into growth medium from pre-culture slants. The experiments were performed on orbital shaker by adjusting the speed 100 rpm at 30 °C for 96 h.

2.4. Solid state fermentation procedure

5 g each of the preliminary processed waste substrate was taken into Erlenmeyer flasks with working volume of 100 mL of the nutrient media. All of the experimental flasks were sterilized and inoculated with 5 mL of inoculum (fungal spore suspension) of *A. oryzae*. All of the freshly inoculated flasks were incubated at 30 ± 1 °C in a shaking incubator at 120 rpm for 5 days of fermentation period. After every 24 h the crude pectin lyase was extracted by mixing the fermented material with 100 mL of 100 mM citrate buffer followed by keeping them in continuous shaking position (120 rpm) for half an hour. The biomass was then filtered by passing through 125 mm filter paper followed by centrifugation at 3000 *g* for 10 min at 4 °C. The resulting supernatants were used as crude extract for enzyme activity estimation. The weight of biomass was determined in pre weighted petri plates and kept in oven for 24 h at 100 °C, till constant weight is obtained.

2.5. Determination of pectinase activity

A UV–Vis spectrophotometric dinitrosalicylic acid reagent (DNS) based method was adopted to determine the pectin lyase activity (Miller, 1959). All of the recorded enzyme activities were expressed as U/mL while, a unit of pectinase activity was defined as a total amount of enzyme required to release 1 μmol of galacturonic acid per mL/min under standard assay conditions.

2.6. Optimization of SSF culture conditions

In the present study a thematic attempt was made to investigate the effect of various parameters including moisture levels, pH, incubation temperatures, carbon sources as an additional growth supplements (glucose, fructose, sucrose, maltose, molasses), nitrogen sources as an inexpensive growth supplements (urea, yeast extract, beef extract, bacteriological peptone, ammonium sulfate), and inoculum size on pectin lyase production in SSF of lemon peel waste (selected from initial screening trial experiment) as most suitable substrate for pectinolytic enzymes production from *A. oryzae*.

Table 1 – Activities of pectin lyase produced by *A. oryzae* on different agro-industrial waste substrates.

Substrates[a] (5 g)	Fermentation time (Days)									
	1	2	3	4	5	6	7	8	9	10
WSW	18 ± 0.9	26 ± 0.6	42 ± 0.2	18 ± 1.5	32 ± 0.2	11 ± 2.6	10 ± 1.2	8 ± 0.7	6 ± 0.2	0 ± 0.3
APW	35 ± 1.2	42 ± 0.5	78 ± 1.5	65 ± 1.6	99 ± 2.2	111 ± 1.2	155 ± 1.8	185 ± 3.3	52 ± 2.3	22 ± 0.0
LPW	145 ± 2.2	199 ± 1.3	288 ± 2.6	313 ± 6.5	274 ± 2.0	238 ± 1.2	199 ± 3.6	155 ± 1.9	99 ± 0.2	66 ± 3.6
OPW	99 ± 3.2	145 ± 3.5	188 ± 2.6	255 ± 2.4	266 ± 3.2	290 ± 4.5	222 ± 3.4	159 ± 2.6	82 ± 1.1	36 ± 1.2
MPW	49 ± 1.2	79 ± 0.3	91 ± 0.6	122 ± 1.0	177 ± 0.2	142 ± 3.6	121 ± 1.5	139 ± 3.1	99 ± 3.2	59 ± 0.1

[a] WSW, Wheat straw waste; APW, Apple pomace waste; LPW, Lemon peel waste; OPW, Orange peel waste; MPW, Municipal paper waste.

2.7. *Statistical analysis*

All of the experiments were conducted in triplicate and S.E. values have been displayed as Y-error bars in figures. Statistical analysis was performed using the statistical software STATISTICA version 8.1.

3. Results and discussion

3.1. *Screening of fermentation time and substrates*

A. oryzae was cultured in SSF medium based on various agro-industrial residual substrates for the production of pectin lyase. After every 24 h a set of experimental flasks were harvested and cultured supernatants were analyzed for enzyme activity assay. The enzyme activity profile obtained in the initial time screening trial showed that maximum production of pectin lyase (313 ± 6.5 U/mL) after 4th day of incubation on lemon peel waste followed 290 ± 4.5 U/mL and 185 ± 3.3 U/mL after 6th and 8th day of incubation on orange peel waste and apple pomace waste, respectively. While using other substrates the enzyme production potential was substantially decreased. The results obtained are summarized in the Table 1. Results of screening trial experiment showed that micro-organism takes some time in settling on the substrate and then showed its maximum activity after stipulated time period. On different substrates pattern of fungus growth may vary because of the difference in composition nature of each substrate. The length of the microbial lag phase and primary metabolism that varies with the chemical composition of lignocellulosic substrates has strong influence on the time taken by microorganism for enzyme synthesis (Asgher, Iqbal, & Asad, 2012; Sen, Veeranki, & Mandal, 2009; Zadrazil, Gonser, & Lang, 1999). Any further increase in the fermentation period caused the production of pectin lyase to decrease. It is also dependent on the nature of fermentation medium, organisms, concentration of nutrients, and the process of physiological conditions (Joshi, Parmar, & Rana, 2006).

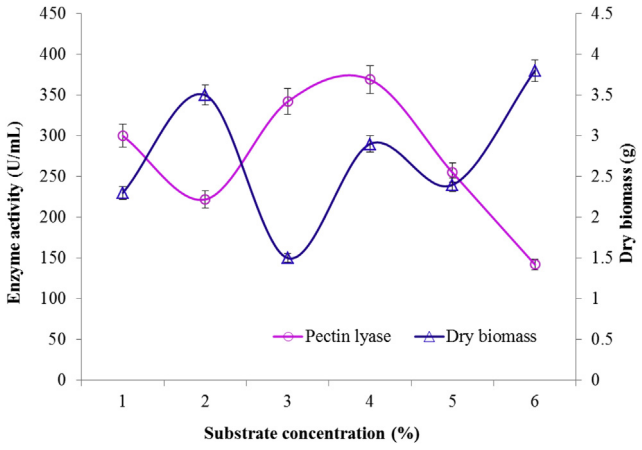

Fig. 1 – Effect of varying substrate concentrations on pectin lyase from *A. oryzae*.

3.2. *Optimization of SSF culture conditions*

3.2.1. *Optimization of substrate concentrations*

Followed by the selection of best yielded growth substrate the optimization of different substrate concentrations (1–6%) on the production of *A. oryzae* pectin lyase was investigated and the results obtained are presented in the Fig. 1. The maximum enzyme activity *i.e.*, 369 ± 5.65 U/mL was observed under optimum (4%) substrate concentration. After careful analysis during the experiment, it was observed that under optimum substrate concentration, more inducers were available to *A. oryzae* that enhance the production of pectin lyase. On the other hand, the present investigation is in contrary to Sathya, Naidu, and Panda (1998), who reported that high concentrations of carbon may inhibit the process of enzyme synthesis. Moreover, the culture organism achieved maximum yield of pectin lyase by hydrolyzing polysaccharides. As a result, the amount of reducing sugar tends to increase when *Aspergillus niger* is used as an enzyme-producing microorganism (Pericin, Madjarev, Radulovic, & Skrinjar, 2007).

3.2.2. *Optimization of moisture level*

An optimum moisture level is a crucial factor in the SSF processes therefore an optimal moisture level is required to get maximum yield of the product of interests. While optimizing this parameter varying levels of moisture (% w/w) were employed ranging from (50–75%) to the growth supported substrate *i.e.*, lemon peel waste. Lemon peel waste fermented at 70% (w/w) moisture gave maximum production (pectin lyase activity; 545 ± 4.85 U/mL) after 4 days of inoculation with *A. oryzae* however, an increase in moisture level from 70% showed decreasing trend in the pectin lyase production (Fig. 2). It has been documented in literature that higher moisture contents had inhibitory effect on microbial growth and enzyme secretion during secondary growth phase (Raghavarao, Ranganathan, & Karanth, 2003). Low moisture contents cause slower enzyme secretion from fungus due to

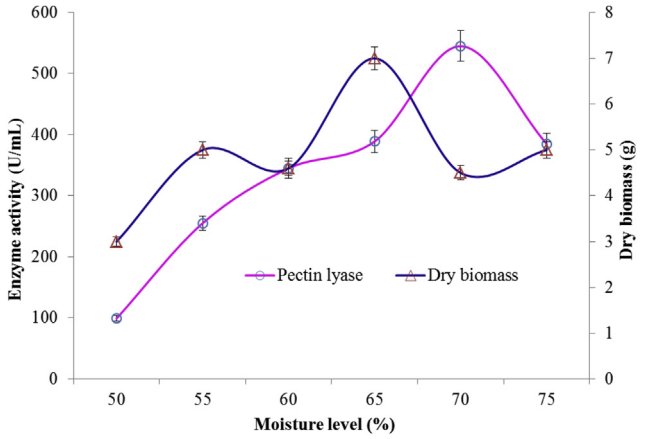

Fig. 2 – Effect of varying moisture levels on pectin lyase from *A. oryzae*.

the lower solubility of nutrients and low level of growth substrate swelling whereas, high moisture level results in decreased substrate porosity that leads to fungal growth inhibition (Iqbal et al., 2011).

3.2.3. *Optimization of pH*

A fermentation medium with varying pH values (2–7) was used to moist the growth supported substrate, lemon peel waste, for the production of pectin lyase. The maximum activity 618 ± 8.98 U/mL was recorded in the SSF media containing lemon peel waste processed at pH 5. From the growth pattern of fungus and culture extracts it was observed that the enzyme formation progressively increased with an increase in the pH of fermentation media and maximum activity was recorded at pH 5 (Fig. 3). Fermentative microorganisms are very susceptible to the variation in the media pH level and also dependent on the physio chemical and nutritional composition of the supplementary growth substrates (Radha, Regupathi, Arunagiri, & Murugesan, 2005). A decline in enzyme activity was observed as the pH level increased from 5. The amount of reducing sugars in the medium released was 3.54 ± 0.9 mg/mL. According to Spagna, Pifferi, and Gilioli (1995), maximum pectinase activity occurred at pH 5.0 while, according to Pedrolli, Gomes, Monti, and Carmona (2008), a pH of 4.5 was the optimum.

3.2.4. *Optimization of incubation temperature*

To investigate the influence of a range of temperatures on the pectin lyase production, shake flask experiments were run at various temperatures between 25 and 45 °C. Maximum enzyme activity 699 ± 5.65 U/mL was observed in the batch culture fermented at 35 °C (Fig. 4). Any further increase in temperature suppressed the enzyme activity. The results obtained in the present investigation are in line of strong agreement with the data reported earlier by Pedrolli et al. (2008) and Nighojkar, Phanse, Sinha, Nighojkar, and Kumar (2006). Temperature above 40 °C results in moisture loss of the substrate which affects metabolic activities of the microorganism that results in reduced growth and enzyme production.

3.2.5. *Optimization of carbon and nitrogen sources*

Completely Randomized Design (CRD) was used to investigate the effect of additional carbon and nitrogen sources as growth supplements. Each of the additional carbon and inexpensive nitrogen supplement was used in different interaction to achieve maximal production of pectin lyase from lemon peel waste. CRD was also applied to study their stimulatory/inhibitory effects under pre-optimized conditions on the production of pectin lyase. The results obtained are summarized in the Table 2. It was recorded that the pectin lyase production pattern changed by the addition of carbon and nitrogen supplements and the combination of glucose and yeast extract gave maximal pectin lyase production 795 ± 6.2 U/mL. The primary carbon and nitrogen source and their valued concentrations are powerful factors that potentially regulate the synthesis of enzymes by fungi under suitable fermentation conditions (Iqbal et al., 2011; Mikiashvili, Elisashvili, Wasser, & Nevo, 2005). Different fungal culture show different growth behavior and enzyme secretion patterns with additional carbon supplements. Some grow better under carbon and nitrogen limited environments but others

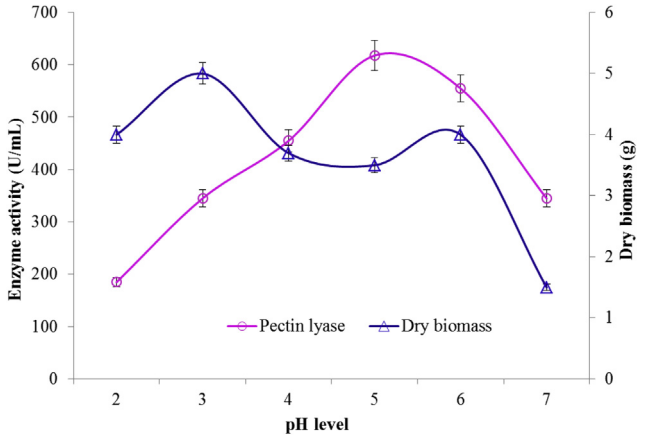

Fig. 3 – Effect of varying pH levels on pectin lyase from *A. oryzae*.

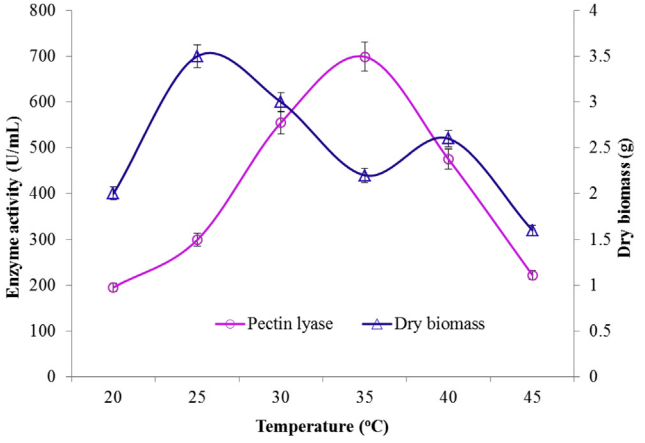

Fig. 4 – Effect of different temperatures on pectin lyase from *A. oryzae*.

Table 2 – Activities of pectin lyase produced by *A. oryzae* under different carbon and nitrogen sources.

Nitrogen sources[a]	Carbon sources				
	Glucose	Sucrose	Fructose	Maltose	Molasses
Urea	388 ± 8.5	654 ± 7.5	455 ± 5.8	484 ± 6.9	327 ± 3.6
YE	795 ± 6.2	722 ± 8.1	554 ± 5.6	421 ± 7.5	389 ± 4.5
BE	525 ± 4.5	655 ± 4.5	456 ± 4.6	658 ± 6.5	455 ± 6.5
BP	666 ± 3.2	422 ± 9.5	654 ± 4.6	555 ± 4.5	342 ± 5.5
AS	718 ± 5.5	555 ± 6.4	354 ± 6.6	457 ± 6.4	285 ± 3.6

[a] YE, Yeast extract; BE, Beef extract; BP, Bacteriological peptone and AS, Ammonium sulfate.

grow better in culture media contained an adequate amount of carbon and nitrogen supplements.

3.2.6. *Optimization of inoculum size*

SSF media containing 5 g lemon peel waste was inoculated with varying inoculum density levels ranging from 1 to 6 mL and incubated under previously optimized fermentation conditions. A maximum pectin lyase activity was recorded in the experimental flasks inoculated 5 mL of fungal inoculum (Fig. 5). An upward trend in the pectin lyase activity was observed with an increase in the inoculum density level from 1 to 5 mL. Fig. 5 illustrating this upward and then downward trend in the activity profile with the inoculum density level from 1 to 5 mL and 5 to 6 mL, respectively. This is may be because a lower inoculums density level (1–3 mL) is not enough for the culture organism to promote the fungal growth resulting in longer lag phase. The reduction in the activity at higher inoculum density is because of the faster depletion of the available nutrients (Iqbal et al., 2011; Patel, Gupte, & Gupte, 2009). Optimum inoculum density is an important consideration for fermentation process since the accumulation of spores can inhibit growth and development of the culture organism (Nighojkar et al., 2006).

4. Conclusions

In summary, *A. oryzae* showed considerable prospective for the production of industrially important pectin lyase in SSF of lemon peel waste (residual waste from citrus field crop) with maximal 875 ± 6.2 U/mL activity. The promisingly high activities of pectin lyase suggest an opportunity of commercialization of the production process using cheap and easily available fermentation growth substrate. In conclusion and the data described above suggests a successful attempt was made to investigate the influence of various physio chemical and nutritional variables on the enzyme production process.

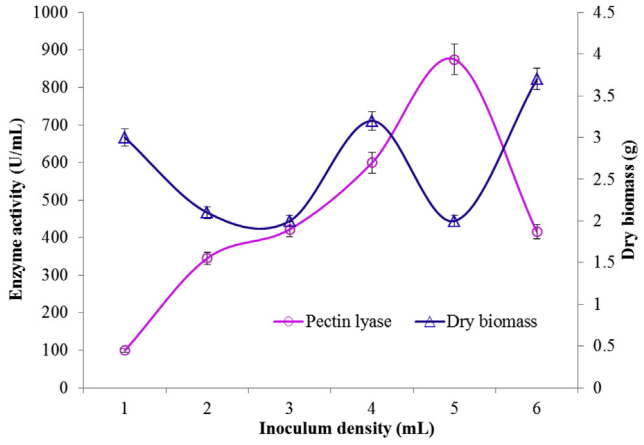

Fig. 5 – Effect of varying inoculums density on pectin lyase from *A. oryzae*.

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