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Cost-effective production of pectinase by parametric optimization of *Pseudozyma sp.* SPJ under submerged fermentation

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ABSTRACT

Pseudozyma sp. SPJ was isolated for the production of pectinase and various culture conditions were standardized to have cost effective production. Maximum pectinase titer was achieved at 35°C for 22 h of incubation. A production broth containing 0.8% citrus peel, 0.125% yeast extract, 0.125% peptone and 10mM $CaCl_2$ (pH 8.0) was found to stimulate excellent (653.13 ± 19.32 U ml^{-1}) enzyme production. Optimum inoculum size was found to be 2% when it was 14 h old. Xylose and lactose were inhibited the enzyme production. Heavy metal ions such as Hg^{2+}, Cu^{2+}, Pb^{+}, Fe^{2+} and Co^{2+} adversely affected the pectinase production. The enzyme production was successfully scaled up at laboratory level. An appreciable amount of pectinase was produced by a newly isolated yeast strain with a cost effective medium under sub-merged fermentation.

KEYWORDS

Pseudozyma sp.;
SPJ;
Pectinase;
Submerged fermentation;
Citrus peel;
Agro-industrial residues.

INTRODUCTION

The biotechnological potential of pectinolytic enzymes from microorganisms has drawn a great deal of attention worldwide because of their myriad applications. Pectinases are the enzymes that breakdown the glycosidic bonds of the long chain of galacturonic acid residues of pectic substances (the structural polysaccharides of plant cell wall). Microbial pectinases contribute to almost 25% of the global food enzyme sales and is estimated to increase further. Pectinases are meant for extraction, liquefaction and clarification of fruit juices[1]. In fabric industry, they are employed to ret plant fibers such as flax, hemp and jute[2]. They are also applied to mechanical pulp to solve the retention problems in the paper industry[3].

Sub-merged fermentation (SmF) is extensively used for the production of enzymes and for understanding the physiological aspects of enzyme synthesis. It involves the growth of the microorganism as a suspension in the liquid medium in which various nutrients are either dissolved or suspended as particulate solids[4]. It is advantageous in terms of easier sterilization and process engineering.

Among the various microorganisms, the reports of pectinase production from yeasts are very few with very low enzyme yield[5,6]. Almeida et al. reported 0.98 U ml^{-1} h^{-1} pectinase titer by immobilized *Kluyeromyces*

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marxianus[7]. Only 17.2 U g^{-1} of activity was reported by Patil and Dayanand using *Aspergillus niger*[8]. *Erwinia, Bacillus, Saccharomyces, Kluyveromyces, Aspergillus, Penicillium, Fusarium* and *Rhizopus* have been the genera most frequently studied in the last 15 years, with strains of *Aspergillus*, *Penicillium* and *Erwinia* mainly used for enzyme production studies[9].

The aim of present investigation is standardization of process variables for the maximum production of pectinase in submerged fermentation of a physiologically potential strain of *Pseudozyma sp.* SPJ isolated from the soil debris of fruit and vegetable markets.

EXPERIMENTAL

Microorganism and its cultivation

An alkaline pectinase producing yeast was isolated from fruit waste disposal site and identified as *Pseudozyma sp.* SPJ by Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, and given accession no. 9842. The microorganism was identified on the basis of its phenotypic characterization and results were further confirmed by 26S rRNA sequencing method. The culture was maintained on yeast extract peptone dextrose (YEPD containing g l^{-1}: Yeast extract, 10.0; Peptone, 20.0; Dextrose, 20.0; Agar, 20.0; pH 6.0) medium slants and stored at 4°C (subcultured every 3 months). Sixteen hour old culture broth grown at 37°C (200 rev/min) was used as seed for inoculation of production broth.

Pectinase assay

Suitable dilution of crude enzyme (10µl) were added to 490µl of pectin (0.1% in appropriate buffer) and incubated at 65°C for 5 min. Then, 1.5 ml of dinitrosalicylic acid (DNSA) was added to the reaction mixture and heated in boiling water bath for 15 min. Absorbance was measured at 540 nm[10]. One unit of enzyme activity was defined as the amount of enzyme that released 1µg of galacturonic acid per min under assay conditions, using galacturonic acid as standard.

Optimization of physiological parameters under Smf

Erlenmeyer flasks (250 ml) containing 50 ml of modified Horikoshi medium were inoculated with 2% (v/V) of 24 h old culture and incubated under shaking conditions (200 rev/min) at temperatures ranging from 25-55°C. After 24 h incubation, the culture broth was centrifuged (10,000 rev/min) for 20 min and the crude enzyme was harvested as supernatant. Pectinase activity was determined in the crude enzyme by Miller's method. Experiments were performed in triplicates and the mean values were taken as results along with standard variation. Similarly the effect of pH (4.0-9.0), Inoculum size (1-4%), inoculums age (12-24 h) and time of incubation (12-30 h) were studied.

Effect of agro-residues as carbon source

Each 250 ml Erlenmeyer flask containing 50 ml of production medium devoid of any carbon source was added with different agro-residues as carbon sources viz. wheat barn, wheat straw, rice bran, rice straw, citrus peel, alpha-alpha leaves and cane baggase (1% w/v). The flasks were inoculated with 1 ml of 14 h old culture and incubated at 37°C, 200 rpm for 22 h. Pectinase activity of the extracted crude enzyme was then determined. The optimum concentration of selected carbon source was determined by using different concentrations of carbon source varied from 0.2-2.0% (w/v) in the medium.

Effect of sugars

Different sugars viz. sucrose, maltose, cellulose, galactose, starch, glucose, xylose, fructose and lactose (0.5% w/v), were added to the Erlenmeyer flasks (250 ml) containing 50 ml of production medium with optimum carbon source. Then they were inoculated and incubated as above. The crude enzyme was extracted and assayed for pectinase activity.

Effect of nitrogen source

Effect of different nitrogen sources (NH_4NO_3, $(NH_4)H_2PO_4$, NH_4Cl, $(NH_4)_2SO_4$, $NaNO_2$, $NaNO_3$, yeast extract, peptone, casein, beef extract, tryptone, KNO_3) on pectinase production was studied by supplementing the medium (0.5%) similarly as that of carbon sources. The concentration of selected nitrogen source was also optimized.

Effect of metal ions

Each 250 ml flask containing 50 ml of production

medium was supplemented with different metal salts (10mM): $BaCl_2$, $MgSO_4$, $MnSO_4$, $FeCl_3$, $FeSO_4$, $CaCl_2$, $CoCl_2$, $ZnSO_4$, Ag_2SO_4, $HgCl_2$, $CuSO_4$, KCl, NaCl, K_2HPO_4, KH_2PO_4, $(CH_3COO)_2Pb$). The flasks were inoculated and incubated as above and assayed for pectinase activity. Thereafter the effect of various concentrations of the selected salt on enzyme production was investigated.

Effect of various additives

Different additives like SDS, EDTA, Tween 20, Tween 80, Triton X 100, Glycerol and Olive oil at a concentration of 2% were supplemented in the medium and their effect was studied.

Laboratory level scale up

Pectinase production by *Pseudozyma sp.* SPJ was attempted to scale up at laboratory level by growing the culture in different sized flasks i.e. 250, 500, 1000 and 2000 ml containing 50, 100, 200 and 400 ml production medium respectively. Each flask was inoculated with 2% culture and incubated (37°C, 200 rev/min and 22 h). Pectinase activity was determined for each flask after 22 h incubation.

RESULTS AND DISCUSSION

Effect of incubation temperature

Pectinase production by *Pseudozyme sp.* SPJ under Smf required optimum temperature of 35°C. Further increase or decrease in temperature resulted in a little decrease in the enzyme activity (Figure 1). The organism was found to grow and produce a good amount of pectinase in a wide range of temperature. The enzyme activity was found to be 87% at 25°C and 72.5% at 55°C which proves it to be potential and thermo-stable. . *Kluyveromyces marxianus* has shown maximum pectinase production at 30°C[7]. However, some yeasts (*Tephrosia candida* and *Kluyveromyces fragilis*) produce pectinases with maximal activities at temperatures up to 60°C[5].

Effect of pH

It is a regulatory parameter in microbiological and biotechnological processes. Maximum pectinase activity (316.84±3.11 U ml^{-1}) was found to be at pH 8.0. Appreciable amount of enzyme was produced in a good range of pH (4-9) as shown in figure 2. Yeasts mainly produce acidic pectinases. The variation in enzyme production due to change in pH may be because of maximum availability of nutrients at that particular pH[11].

Effect of inoculum age & size

The pectinase titer was found to increase when the age of inoculum was increased from 12- 14 h and declined thereafter (Figure 3). It shows a very high growth rate of the organism.

A very little variation was found in pectinase yield when attempted to grow with inoculum 1-4%. Maximum enzyme production (473.09 ± 1.25 U ml^{-1}) was found with 2% inoculums (Figure 4). Higher concentration of inoculum is not preferable in industrial fermentations[12]. It might be due to a competition for nutrients among the yeast population as observed in *Bacillus coagulans*[13] and *Thermomucar indicae-seudaticae*[14].

Optimum time of incubation

The time of incubation depends on the growth rate of the microorganism and its enzyme production pattern. The extracellular levels of pectinase were monitored from 12-42 h. The maximum enzyme activity was observed after 22 h of incubation. It declines after 24 h and stabilizes thereafter (Figure 5). Almeida et al. reported 24h of incubation time as optimum for *Kluyveromyces marxianus for* maximum pectinase production[7].

Effect of different carbon sources

About 30-40% cost of the industrial enzymes are accounted for the substrate used[15]. Thus it is important to select a low-priced substrate to make the enzyme production more economical. The nature of the growth substrate exhibited a significant effect on pectinase production by *Pseudozyma sp.* SPJ. Among various inexpensive and easily available agro-industrial residues tested as sole carbon source, maximum pectinase titer was attained in the medium supplemented with citrus peel followed by wheat straw (TABLE 1). Citrus peel contains 30% (approx.) of pectin in its fresh weight. It is also reported earlier as a very good substrate for pectinase production[16-19]. The difference in enzyme induction can be attributed

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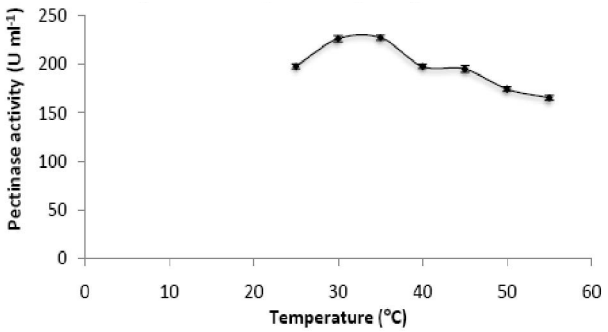

Figure 1 : Effect of incubation temperature on pectinase production by *Pseudozyma sp.* SPJ

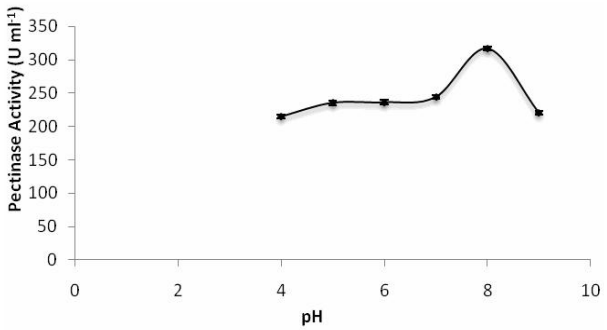

Figure 2 : Effect of pH on pectinase production by *Pseudozyma sp.* SPJ

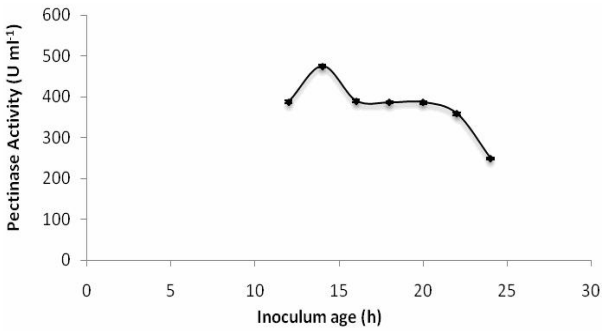

Figure 3 : Effect of inoculum age on pectinase production by *Pseudozyma sp.* SPJ

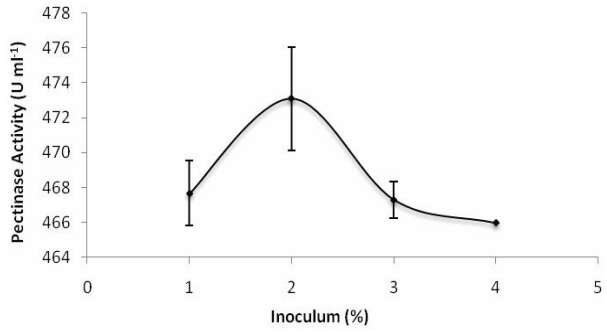

Figure 4 : Effect of inoculum size on pectinase production by *Pseudozyma sp.* SPJ

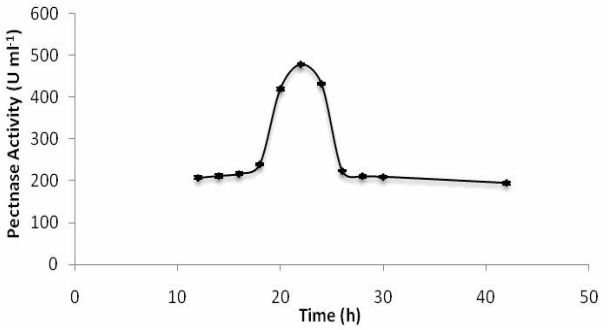

Figure 5 : Effect of time of incubation on pectinase production by *Pseudozyma sp.* SPJ

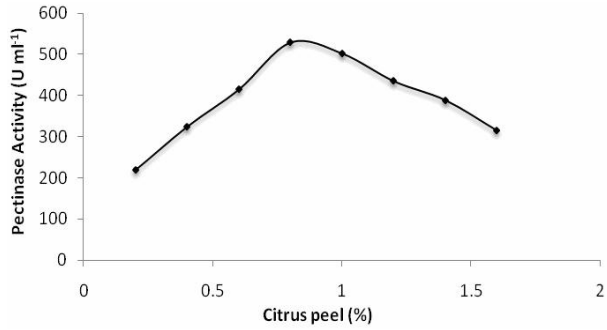

Figure 6 : Effect of Conc. of Citrus peel on pectinase production by *Pseudozyma sp.* SPJ

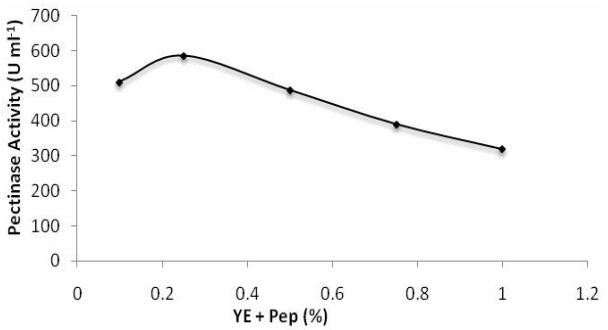

Figure 7 : Effect of Conc. of Yeast extract + Peptone on pectinase production by *Pseudozyma sp.* SPJ

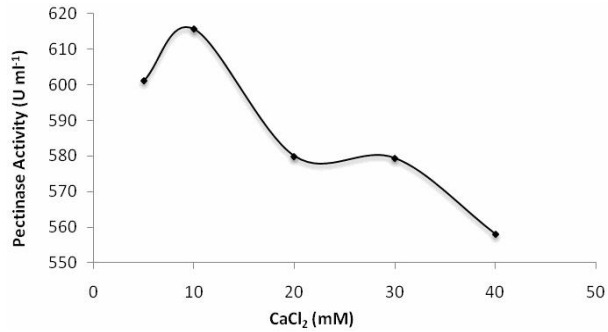

Figure 8 : Effect of Conc. of CaCl2on pectinase production by *Pseudozyma sp.* SPJ

TABLE 1 : Pectinase production on various Agro-industrial residues as carbon source

Carbon source	Pectinase Activity (U ml^{-1})
Control	186.49 ± 1.17
Wheat bran	258.64 ± 2.37
Wheat straw	371.13 ± 2.83
Rice bran	328.84 ± 3.94
Rice straw	340.16 ± 2.87
Citrus peel	474.09 ± 3.67
Alpha-alpha leaves	304.71 ± 2.85
Cane bagasse	237.5 ± 3.54

to the difference in the chemical nature and particle size of the substrates used.

The pectinase activity increased with the increase in concentration of citrus peel from 0.2% to 0.8% w/v and it declined considerably with further increase (Figure 6). This may be due to the formation of a thick suspension of the substrate, which in turn did not mix freely in shake flasks.

Effect of different sugars

No sugar has enhanced the pectinase activity when applied in combination with 0.8% citrus peel in the production broth. However, galactose, lactose and xylose found to repress the pectinase activity when present in the production broth (TABLE 2). Kuhad et al. reported increased pectinase production *Streptomyces sp*. RCK-SC by using lactose, maltose and sucrose[20]. The most plausible explanation for decrease in pectinase production with other carbon sources is that these sources exerted catabolite repression[21].

Effect of different nitrogen sources

Among the various nitrogen sources used a combination of yeast extract and peptone (1:1) was found to be most promising (TABLE 3). This may be because peptone contains essential amino acids, and yeast extract is also a good source of naturally occurring B-complex vitamins[22]. Yeast extract has been reported to be used as best carbon source for *Kluyveromyces cerevisiae*[23]. Yeast extract and peptone have also been reported to stimulate maximum pectinase production from *Kluyveromyces lactis* NRRL 1137[24]. KNO_3 is found to be best among the inorganic sources used.

The highest Pectinase activity was achieved by using 0.25% of Yeast extract and peptone combination i.e. 0.125% each in the production broth. Beyond this concentration, decreased in the enzyme activity was observed (Figure 7).

TABLE 2 : Pectinase production on various sugars in combination with citrus peel

Sugar	Pectinase activity (U ml^{-1})
Control	563.51 ± 11.38
Sucrose	372.51 ± 9.52
Maltose	423.96 ± 7.37
Cellulose	540.33 ± 10.18
Galactose	114.4 ± 5.44
Starch (soluble)	553.4 ± 7.64
Glucose	500.78 ± 8.93
Xylose	98.8 ± 5.63
Fructose	526.11 ± 6.95
Lactose	73.51 ± 3.55

This may be due to the fact that complex nitrogen sources like peptone release NH^{+4} ions, which stimulate growth and increase enzyme yield because of its protease inhibiting nature at lower concentrations but a high concentration of peptone acts as a good inducer for proteases leading to the increased degradation of other enzymes[25].

Effect of different metal ions

Among the different salts tested, $CaCl_2$ was found to enhance the pectinase activity. The effect of calcium on pectinase production is well established[26]. Heavy metal ions such as Hg^{2+}, Cu^{2+}, Pb^{+}, Fe^{2+} and Co^{2+} adversely affected the pectinase production (TABLE 4). Mn^{2+} has inhibited the enzyme activity to almost nil.

The concentration of $CaCl_2$ was studied from 5mM to 40mM (Figure 8). 10mM $CaCl_2$ resulted in maximum pectinase titer from *Pseudozyma sp*. SPJ. High concentration of salt could block the secretion of enzyme into the external medium[27].

Effect of different additives

Among the additives used, SDS and EDTA inhibited pectinase production strongly whereas the maximum increase in pectinase production was observed in the presence of Tween 80 followed by Olive oil (TABLE 5). But the production broth devoid of any additive was near to the maximum. Therefore, no supplement is required in order to increase the pectinase activity.

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TABLE 3 : Effect of different nitrogen sources on pectinase production

Nitrogen source	Pectinase activity (U ml^{-1})
Control	368.36 ± 4.26
NH_4NO_3	355.98 ± 7.39
NH_4H_2PO4	365.04 ± 8.57
NH_4Cl	367.64 ± 6.99
$(NH_4)_2SO_4$	353.53 ± 10.29
$NaNO_2$	203.11 ± 2.77
$NaNO_3$	349.6 ± 5.61
Yeast extract	471.76 ± 7.25
Peptone	468.53 ± 9.17
Yeast ext. + peptone	588.67 ± 8.91
Caesin	356.13 ± 4.21
Beef extract	226.02 ± 4.45
Tryptone	179.02 ± 5.93
KNO_3	468.78 ± 6.53

TABLE 5 : Effect of various additives on pectinase production

Additives	Pectinase activity (U ml^{-1})
Control	651.67 ± 20.03
Tween 20	610.47 ± 18.97
Tween 80	653.13 ± 19.32
Triton X 100	495.11 ± 12.22
Olive oil	644.24 ± 18.43
Glycerol	551.07 ± 11.96
SDS	252.89 ± 5.38
EDTA	182.53 ± 2.29

TABLE 4 : Effect of different metal salts on pectinase production

Metal salts	Pectinase activity (U ml^{-1})
Control	476.14 ± 8.57
KCl	540.78 ± 13.76
NaCl	506.36 ± 9.62
$(CH_3COO)_2Pb$	283.36 ± 10.39
$MgSO_4$	510.02 ± 6.92
$FeSO_4$	274.58 ± 5.83
$FeCl_3$	222.87 ± 3.74
$HgCl_2$	294.33 ± 3.42
Ag_2SO_4	316.78 ± 8.31
$ZnSO_4$	332.07 ± 6.73
$MnSO_4$	51.06 ± 2.89
$CuSO_4$	287.42 ± 4.13
$CoCl_2$	278.87 ± 3.57
$CaCl_2$	594.8 ± 10.48
$BaCl_2$	489.51 ± 11.92
K_2HPO_4	449.93 ± 7.76
KH_2PO_4	457.13 ± 9.38

TABLE 6 : Laboratory level scale up of pectinase production by *Pseudozyma sp.* SPJ

Production broth volume (ml)	Pectinase activity (U ml^{-1})
50	651.0 ± 3.96
100	630.38 ± 2.98
200	620.2 ± 3.66
400	600.2 ± 2.73

The use of surfactants and fatty acids is well documented to increase the production of hydrolytic enzymes[28,29]. The inhibition of enzyme activity by EDTA can be attributed to its binding to the metal ions which are necessary for the enzyme action. A little inhibition shows the potential of the enzyme for its application in flax retting and plant DNA extraction where EDTA is used as chelating agent[30,31]. Such compounds probably increase the permeability of the cell membranes and cause rapid secretion of the enzymes. SDS may inhibit the enzyme activity by denaturing the enzyme structure.

Laboratory level scale up

An attempt to scale up pectinase production was made under laboratory conditions. The yield decreases (651.0 ± 3.96 U ml^{-1} to 600.2 ± 2.73 U ml^{-1}) when the volume of the flasks increases from 250 ml to 2 L (TABLE 6). A negligible decline was observed with the increase in volume of the production medium which makes it a potential candidate for industrial applications.

CONCLUSION

The present investigation concludes that parametric optimization of pectinase production by *Pseudozyma sp.* SPJ has improved the enzyme activity by about 2.8 times. The enzyme production using a low cost medium makes it more fruitful for industrial sector.

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