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Production and Optimization of Cellulase Enzymes from Newly Isolated Fungi

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The main purpose of this study to reduced the production cost of cellulase by using alternative carbon source such as agricultural waste residue and optimized fermentation medium for high yielding. The present investigation was carried out to isolate the novel cellulase producing fungi, CPF-1 & CPF-2 with optimization of physicochemical and nutritional parameters for cellulase production. Among agricultural residues, rice bran and wheat straw were found to be best carbon source for the production of cellulase by CPF-1 and CPF-2, respectively. The optimal parameter (Temp, pH and incubation time) for the production of cellulase by CPF-1 and CPF-2 were 28^{0}C, pH 6 & 120 min and 28^{0}C, pH 7 & 96 min respectively. Also the thermal stability of both the purified cellulase enzymes was high at 60^{0}C for 85min & 70 min for CPF-1 and CPF-2 fungi respectively.

Key words: Agricultural waste, Biodegradation, Cellulase, Fermentation, Optimization.

Cellulose is the most abundant and renewable biopolymer on earth. It is the dominating waste material from agriculture. It constitutes the waste generated from both natural and man made activities. The need for utilizing renewable resources to meet the future demand for food and fuel has increased the attention on cellulose as the only foreseeable sustainable resource of fuel available to humanity. Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa.

In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol (Olson, Hahn-Hagerdahl, 1997; Levy *et al*., 2002; Van Wyk, Mohulatsi, 2003), organic acids (Luo *et al*., 1997), detergents and other chemicals (Oksanen *et al*., 1998). They have been used in the pulp and paper industry, e. g., in deinking of fiber surfaces and in improving pulp drainage (Oksaren *et al*., 2000; Suurnakki *et al*., 2004), in the textile industry (Cavaco-Paulo, Gubitz, 2003; Nierstrasz, Warmoeskerken, 2003; Miettinen-Oinonen *et al*., 2004), animal feed (Ishikuro, 1993), and even in the food industry (Penttila *et al*., 2004; Urlaub, 2002), for the processing of paper and cellophane, as well as for biotransformation of waste cellulose to fermentable sugars (Van Wyk, Mohulatsi, 2003). As lytic enzymes, they are of prime importance is the protoplast production (Davis, 1985; Mandels, 1974; Bhat, 2000). The demand for more thermostable, highly active and specific cellulases is on the increase.

Fungi are well-known agents of decomposition of organic matter in general and cellulose substrates in particular (Lynd *et al*., 2002). Fungal cellulases are inducible enzymes that are

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usually excreted into the environment (Bhat, 1997) and depend on cellulose type (amorphous or crystalline) acting on the organism (Ortega *et al*., 2001).

This study concentrates mainly on isolation of efficient cellulase producing fungi and fungal ability to utilize various agricultural residues as a carbon source.

MATERIALS AND METHODS

The Wheat straw was collected from the farm of Wheat. The Rice bran was collected from Rice Mill. The Saw dust was collected from the timber mill. The coir waste was collected from near by temple area and the Orange peel was collected from juice industry.

Isolation

Strains were isolated from the agricultural waste dumped on farm and agroindustry. Strain isolation was done by serial dilution method on Carboxy Methyl Cellulose-Agar plate.

Pretreatment

The bio waste substrates were sun dried and crushed. The crushed substrates were then sieved individually to get power form. Then the substrate were soaked individually in 1% sodium hydroxide solution (NaOH) in the ratio 1:10 (substrate:solution) for two hours at room temperature. After incubation the treated substrates were filtered and autocalved at 121°C for 1h (Arunkumar *et al*, 2009).

Nutrient

Nutrients described by Mandels and Weber, 1969; for cellulase productions were supplied in concentrated form, but proteose peptone was replaced with yeast extract. The required amount of concentrated nutrient salt solution was added to 5 g of substrate. The concentrated Czapek—mineral salt agar medium were dissolved in 200 mL of water and autoclaved.

Inoculum preparation

It was prepared from a 7 days old slant by adding 10 mL of sterilized 0.8% Tween 80 to it. The spores were scratched with the help of a sterilized wire loop to make a homogeneous suspension of spores. The suspension is used as inoculums (10^7/ml) Spore count was done with the help of Haemocytometer.

Extraction of the cellulase system

The culture of SSF from each flask (originally 5 g of substrate) was mixed well with more water to bring the final weight of the mixture (mycelium plus unutilized lignin, cellulose, and hemicelluloses) to 100 g. Tween 80 was added at a rate of 0.1%. The mixture was shaken for 30min and centrifuged. The supernatant was used for enzyme determination (Chahal, 1985).

Ammonium sulphate precipitation

The proteins in the crude preparation were precipitated by the addition of solid ammonium sulphate to 80% saturation. The Fermentation flasks were added adequate amount of sterile distilled water and Tween 80 to extract the cellulase which is absorbed on the residues (mycelium and unutilized cellulose, hemicelluloses and lignin).Then the Mixture is was kept under shaking condition several time to release the absorbed cellulase. The supernatant was collected and stored at 4°C.The ammonium sulphate was weigh exactly 51.6 g and crushed to convert in to power form. The ammonium sulphate precipitation was started by adding pinch of powder of ammonium sulphate in the supernatant at a time. It is to be noted that the precipitation must be carried out in continous shaking condition at 0°C. After dissolving whole powder, the precipitates are kept overnight at 4°C. The precipitates were collected by cold centrifuge at 10,000 rpm. The supernatant was discarded and the pellets were collected and dissolved in the sodium citrate buffer (pH 4.8) and stored at 4°C.

Analytical method

Cellulase activity was analyzed by the filter paper assay. 50mg of Whatman No.1 filter paper (~6*1cm strip) was incubated with 0.5ml of the enzyme preparation and 0.5ml of phosphate buffer for 1h at 50^0C. The glucose liberated was estimated by DNS method. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1µg of glucose per minute under the standard assay conditions.

Optimum temperature

For estimation of the optimum temperature of the enzyme, the activity was determined by carrying out the assay at several temperatures between 30°C and 80°C.

Optimum pH

The pH profile of the enzymes was evaluated by incubating the enzymes in Sodium

citrate buffer at different pH 4.5, 5.5, 6.5, 7.5 and 8.5.

Heat stability

The thermo stability of the enzyme was determined by incubating the enzyme for three hrs at particular temperature.

RESULTS AND DISCUSSION

Isolation and screening of CPF

More than 10 newly cellulase producing fungi (CPF) were isolated. During screening procedure with 1% congo red staining solution, two best clear zone showing CPF was screened out and go for further analysis (Fig 1.b & 2.b). as shown in, fig 1.a,c & 2.a,c both the fungi strain were distinct from in each other according to colonial and morphological characteristics.

Effect of various substrates on enzyme production

Data presented in fig-3 shown that cellulase production by CPF 1 and CPF 2 were significantly influenced in rice bran and wheat straw respectively. Results indicated that CPF showed higher 4.22 (IU/ml) activity in rice bran and CPF 2 in wheat straw like 3.7 (IU/ml). Cellulase production commended on reaching nitrogen limiting conditions and the yield of cellulase decreased when excess peptone was presented, various inorganic nitrogen sources have been optimized by different workers for cellulase production (Sherief *et al*. 2010~ Solomon *et al.* 1997~ Lee *et al.* 2010).

Effect of pH on enzyme production

Cellulase yield by CPF 1 and CPF 2 appear to depend on pH value. Results illustrated by Fig 4 clearly show that cellulase production by CPF 1 and CPF 2, expressed high enzyme activity 3.24 and 3.12 (IU/ml) at pH 6 and 5, respectively.

Effect of temperature on enzyme production

Temperature is also an important factor that influences the cellulase yield. Maximum enzyme production by CPF 1 and CPF 2 were found to be 3.04 and 2.99 (IU/ml) activities at 28 ^{0}C (Fig 5). Many workers have reported different temperatures for maximum cellulose production either in flask or in fermenter studies using *Trichoderma* sp. suggesting that th optimal temperature for cellulase production also depends on the strain variation of the microorganism (Murao *et al*., 1988, Lu *et al*., 2003).

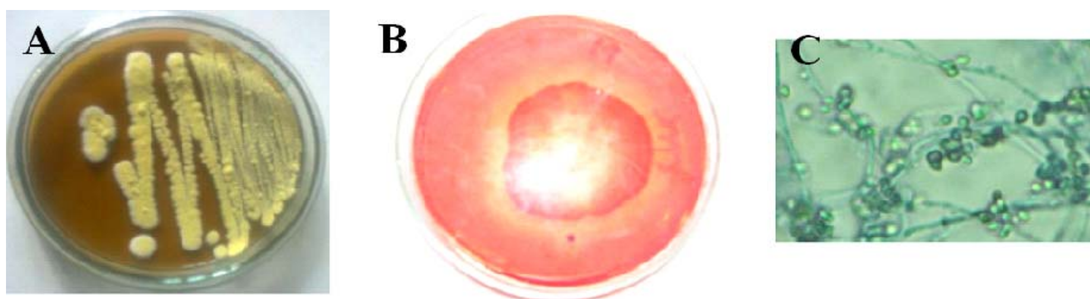

Fig.1. Morphological Characteristics of CPF 1

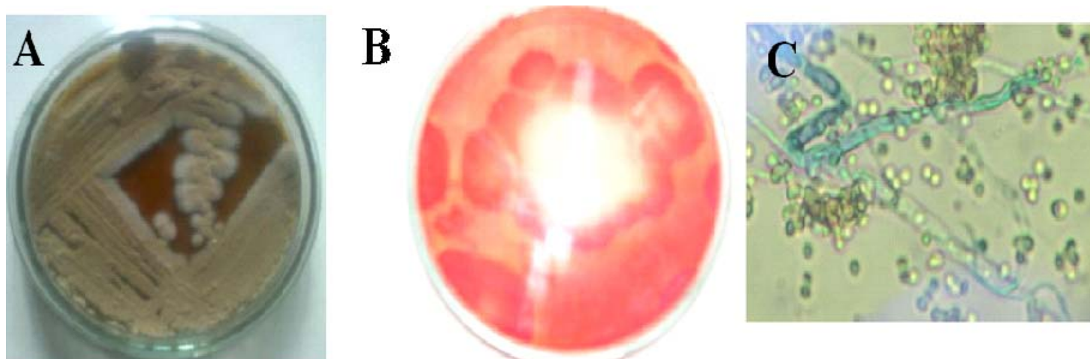

Fig. 2. Morphological Characteristics of CPF 2

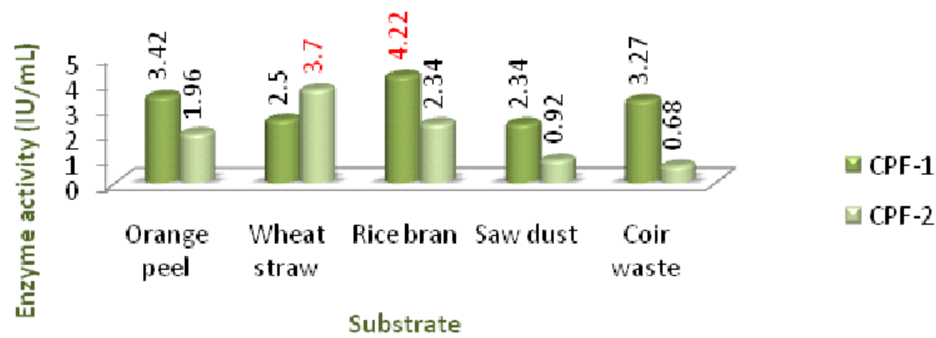

Fig. 3. Enzyme production at various substrate

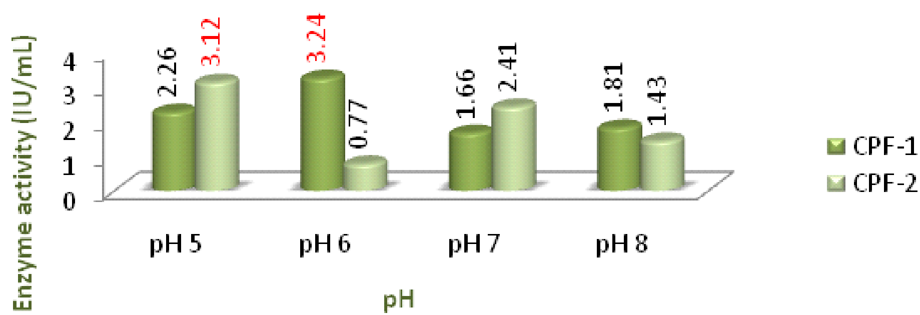

Fig. 4. Enzyme production at various pH

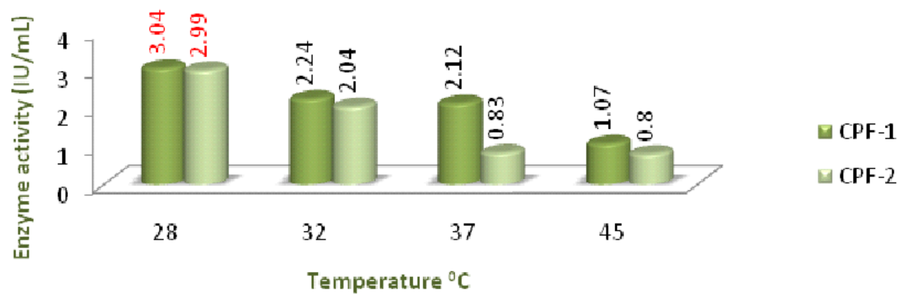

Fig. 5. Enzyme production at various Temperature

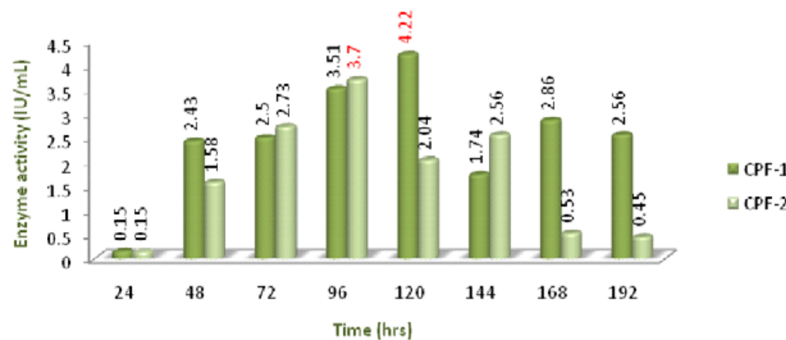

Fig. 6. Enzyme production at different incubation time

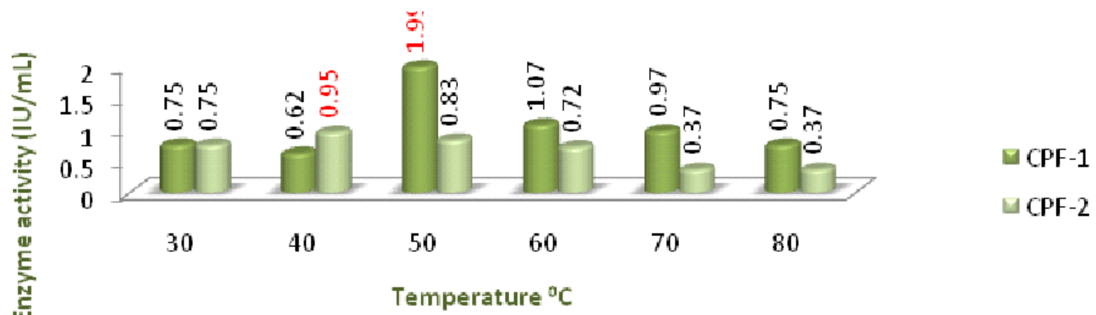

Fig. 7. Optimum temperature for enzyme activity

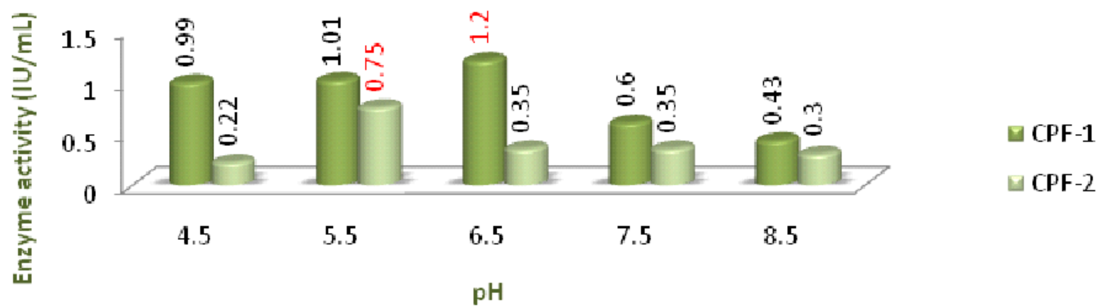

Fig. 8. Optimum pH for enzyme activity

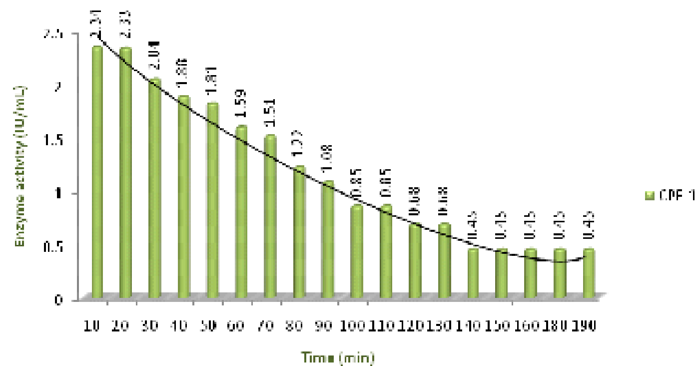

Fig. 9. Termal stability of CPF-1 Enzyme

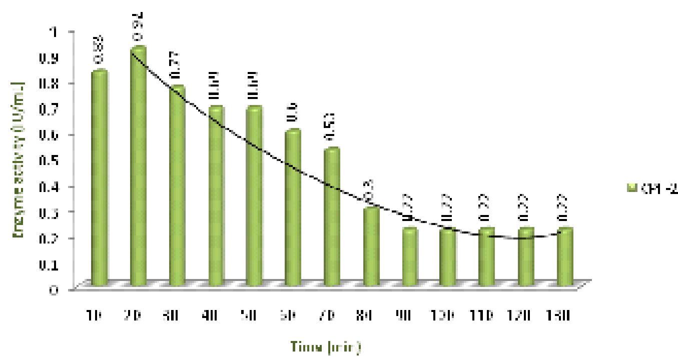

Fig. 10. Termal stability of CPF-2 Enzyme

Effect of incubation period on enzyme production

The incubation period is directly related with the production of enzyme and other metabolic up to a certain extent. Fig. 6 indicated that CPF 1 and CPF 2 showed higher cellulase activity 4.22 (IU/ml) and 3.7 (IU/ml) during 120 hrs and 96 hrs, respectively. It might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes (Nochure *et al*., 1993).

Optimum temperature for enzyme activity

After purification of enzymes, its activity is to be estimated at various temperatures. Fig 7 clearly indicated that CPF1 enzymes shows higher activity at 50^0C, while CPF 2 enzymes show higher activity at 40^0C. According to industrial point of view, enzymes are very much useful in bioprocessing, which are show higher activity at high temperature. So, here CPF1 enzyme is more beneficial then CPF2 enzymes.

Optimum pH for enzyme activity

Low pH working enzyme is very much beneficial for industry in downstream processing. Fig 8 indicated that CPF 2 (0.75 IU/ml) shows good activity compared to CPF 1 (1.2 IU/ml) at low pH.

Thermal stability of enzymes

Fig 9 & 10 indicated that both enzymes show higher thermal stability to 90 min and 60 min at 50 ^{0}C by CPF1 and CPF 2 enzymes, respectively. This results show good impact on juice, paper, pulp and sugar industries, where high thermal stability of enzymes required for long period of time.

CONCLUSION

Production and optimization of cellulase has been found suitable for CPF 1 and CPF2 in different agro waste. Also the optimization of pH, temperature, incubation time is major limiting factor for maximum cellulase enzyme production as well as enzymes activity. The present study evaluated the application of different agro industrial residues as substrates for cellulase production. This study provides the cost effective technology for the production of enzymes and SSF is a suitable technology for economical production of cellulases using lignocellulosic residues as substrate.

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