Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Special Issue [1]2022 : 1075-1080

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Halotolerant protease enzyme production by *Penicillium chrysogenum* from Marakkanam Saltpans, Tamil Nadu, India

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ABSTRACT

Halophiles are seen as an admirable source of novel enzymes having natural capacity to operate under saline and hypersaline environment conditions. The article covers and puts in perspective the isolation, optimization and partial purification of protease from halophilic fungi of Penicillium chrysogenum. Twenty one fungal strains were isolated from sediment sample and these isolates were screened for the production of protease enzyme. Among them, a strain P. chrysogenum, which produced highest protease activity in primary screening in Casein agar media and this strain, was selected for further study. The highest protease enzyme could be achieved using an optimized medium of the following incubated at shaking condition (agitation speed 100 rpm), pH 9, temperature 50°C, the best skim milk, ammonium nitrate and 4% of NaCl concentration that provided the highest enzyme production from P. chrysogenum. For purification of protease, 80% ammonium sulphate saturation was found to be suitable giving maximum protease activity. Hence, the halophilic fungi of P. chrysogenum demonstrated promising proteolytic activity for biotechnological applications as a result of this investigation.

Keywords*: Penicillium chrysogenum, Halophiles, protease, optimization*

Received 13.02.2022 Revised 22.03.2022 Accepted 20.04.2022

INTRODUCTION

The search for new enzymes endowed with novel activities and enhanced stability continues to be a desirable pursuit in enzyme research. This is fuelled by industrial requirements and necessity of enzymatic interventions in new and challenging bioprocesses. In this context, enzymes from extremophiles are often useful because they withstand and carry out catalysis under extreme physiological conditions. Extremophiles are therefore perceived as an excellent source of novel enzymes possessing inherent ability to function under extreme conditions [1]**.** Alkaline proteases of halophilic origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment etc [2]. New protease-producing microorganisms and perfected fermentation technology are needed to meet the ever-growing demand for this enzyme. Bioprospecting for new protease producing microorganisms from less studied environments may help to this end.

The adaptation of fungal cells to high saline environments is a promising biological process. Several species of *Aspergillus* have been investigated in detail for the production of alkaline proteases under solid state fermentation condition [3-5]. The genus like *Trichoderma* [6], *Rhizopus* [6] and *Beauveria* [6], *Penicillium*sp. [9, 10] excretes the acid proteases in both submerged and solid state fermentation conditions. *Penicillium charlesii* [11] and *P. griesofulvum* [12] are known to produce alkaline proteases under SMF and SSF condition respectively.

While, the halophilic fungi has been reported by various researchers but further investigation may add its importance and potentialities. A research study has been undertaken to explore its more utility specifically its biotechnological applications. Hence, the present study was aimed to evaluate the production and optimization of protease from halophilic fungi of *Penicilliumchrysogenum*.

MATERIAL AND METHODS

Collection of Samples

Sediment sample was collected from saltpan of Marakkanam, Tamil Nadu, India in a sterile plastic covers and brought to the lab, stored in the refrigerator at 4 °C until it was used.

Isolation of fungi

One gram of collected soil sample was diluted in 99 ml blank and from the dilution 1 ml was serially diluted to the test tubes containing 9 ml of sterile distilled water and dilutions were made up to 10^{-6}. From the each dilution, 1 ml of dilution suspension was pipetted and plated on a sterile PDA. Bacterial contamination was inhibited by adding 0.05% of chloramphenicol in PDA. All plates were incubated at 25ºC for 5-7 days.

Screening for amylase production

Twenty one fungal strains were isolated in Czapeck Dox Broth (CDB) containing 1% casein for the screening of protease production. Sterile 50 ml of CDB medium with 1% casein inoculated with two mycelial discs (8 mm) of a fungal strain and incubated 37^0C in rotary shaker for 6 days. The culture filtrate was separated and centrifuged at 8000 rpm for 15 minutes. Mycelium free culture filtrate obtained was used for the qualitative assay of protease and activity. Water agar medium (14) (1.8g agar in 100ml of 50% Seawater) supplemented with 1% casein were poured in to petriplates and after solidification wells were made using a 8 mm diameter cork borer. 100µl of culture filtrate was poured in one well and same volume of uninoculated medium was added in a well as control and incubated at room temperature for 24 hours. The enzyme activity was visualized as a clear zone on addition of 1 % mercuric chloride solution in 1N HCl. The diameter of zone formed was determined for all the positive strains. Based on it *P.chrysogenum* strain was selected for further studies. The strain was maintained on potato dextrose agar and used for further to study on protease production

Optimization for amylase production

Factors affecting cell growth and α-amylase production were investigated using one factor at a time method. The optimized parameters viz., static and shaken conditions (50 to 250 rpm), carbon source (0.5% of starch, wheat bran, glucose, skim milk and sucrose), nitrogen source (0.5% of beef extract, peptone, gelatin, sodium nitrate and ammonium nitrate), temperature (20°C to 60°C), pH (3.0 to 11.0), salt concentrations (1 to 5 %) were tested

Purification of enzyme

Ammonium sulphate precipitation

Purification steps were carried out at 4ºC. In the initial purification step, the supernatant containing the extra cellular amylase was treated with different saturation levels of solid ammonium sulphate (up to 80% saturation level) as described by Wang *et al.* 2006, with continuous overnight stirring. The precipitated proteinaceous material was collected by centrifugation (10,000 rpm for 15 min) and dissolved in 0.1M phosphate citrate buffer (pH 5.0). The enzyme solution was dialyzed using dialysis membrane No-150 (Himedia) against the same buffer for 48 h with several intermittent buffer changes. The dialyzed protein fraction was lyophilized to a powder. The enzyme activity was assayed in each and every step following the procedure stated previously.

RESULTS

All the isolated 21 fungal strains belonging to nine genus were qualitatively screened by well diffusion assay method using water agar medium supplemented with 1% casein. Based on the diameter of zone of clearance they were classified into three categories *viz*., high enzyme activity (+++), medium enzyme activity (++) and low enzyme activity (+). The strains that failed to produce clear zones were denoted as non producers (-). Among the 21 strains, only 4 namely,*A. fumigatus, A. flavus*, *P. chrysogenum* and *P. citrinum* were belonged to the +++ category, 11 strains of ++, 4 strains of + category and another 2 strains did not exhibit protease activity. Among the +++ category, *P. chrysogenum*exhibited largest zone of casein lysis (9.1 mm). Hence it was selected for further studies on production and optimization of protease enzyme.

Optimization of α-amylase production conditions

Selected strain *P. chrysogenum*was subjected to various culture conditions to investigate the optimum culture conditions for α-amylase production.

Effect of static and shaking conditions on protease production

The results obtained from the static and shaking conditions in submerged culture cleared that the protease productivity was gradually decreased with the increment in the shaking condition. Shaking condition of 100 rpm was the best for protease productivity (174 U/ml of enzyme). Minimum was observed in static as well as at 300 rpm. However the biomass during static conditions was found to be high (0.702 mg) compared to other shaking conditions (Fig 1).

Effect of different carbon and nitrogen sources on protease production

The broth fortified with 05 different carbon sources at the concentration of 0.5 % was inoculated with *P. chrysogenum* and kept in a shaker at 100 rpm for five days. The highest enzyme activity was achieved in sucrose fortified medium (183 U/ml) where the biomass produced was about 0.608 mg (Fig 2). Ammonium nitrate has registered the maximum enzyme activity of about 322 U/ml and the biomass registered was 0.654 mg. Likewise the minimum enzyme activity (175 U/ml) was found in gelatin augmented medium where the biomass was found to be 0.482 mg (Fig.3).

Fig.1. Enzyme activity and biomass in Static/shaken conditions

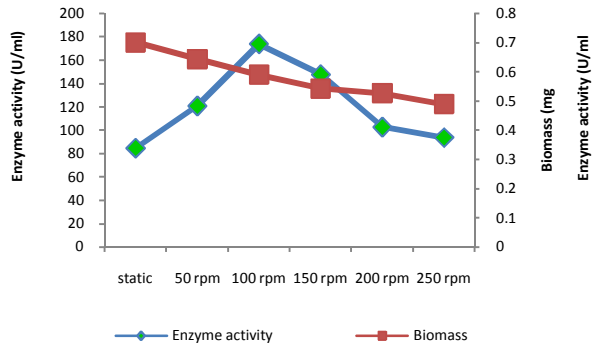


Fig.2. Enzyme activity and biomass in different carbon sources

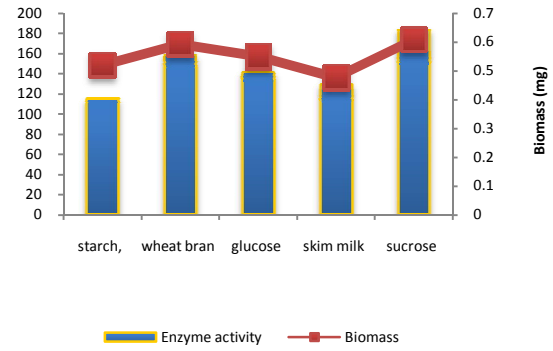
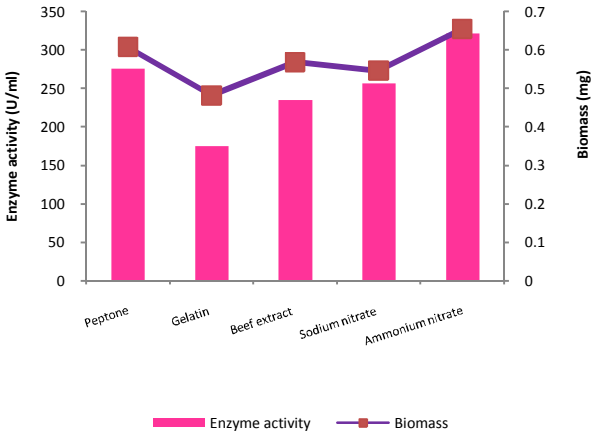


Fig 3: Enzyme activity and biomass in different nitrogen sources



Effect of different pH, temperature and salt concentrations on protease production

The initial medium pH ranging from 3-11 was studied to detect the effect on amylase production by *P. chrysogenum* in MSM broth with previously optimized parameters. The pH 9 supported maximum protease production (*i.e.*) 314 U/ml with the biomass of 0.666 mg. The lowest pH 3 produced minimum amylase activity (24U/ml)even though minimum protease activity (98U/ml) was observed in pH 5 (Fig.4). Optimum temperature for protease production was standardized for which different temperatures ranging from 20°C to 60°C were examined. The result showed the maximum amylase activity at 50^{0}C (398 U/ml) while the minimum was observed at 20°C (108 U/ml). The biomass also found to be maximum at 50°C (0.625 mg) and minimum (0.327 mg) at 20°C (Fig.5). As the fungus *P. chrysogenum* was isolated from a salt environment, the effect of varying percentage of NaCl concentration on amylase activity was studied. At 4% of salt concentration, it gave maximum amylase activity (467 U/ml) and minimum enzyme activity was obtained at 1% of salt concentration (288 U/ml (Fig.6).

Fig 4: Enzyme activity and biomass in different pH

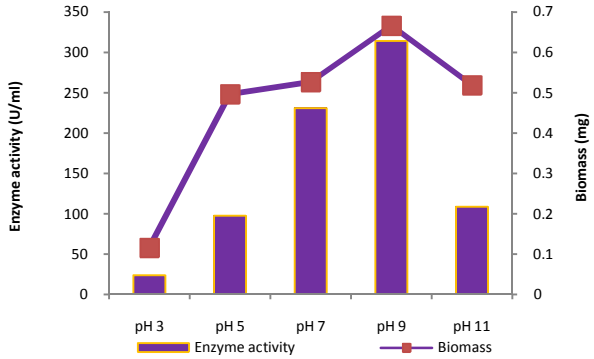


Fig 5: Enzyme activity and biomass in different temperature

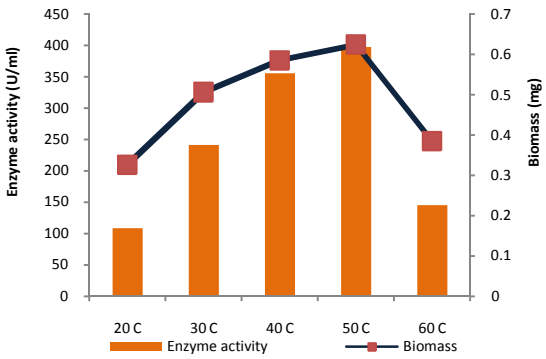
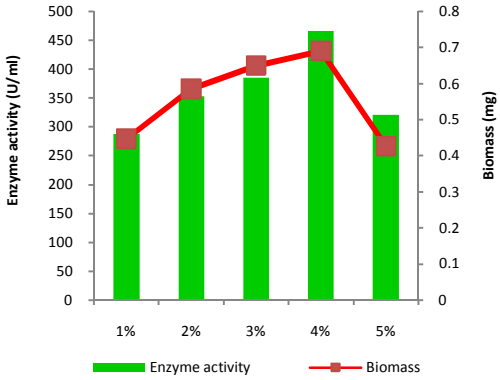


Fig 6: Enzyme activity and biomass in different Salt concentrations



Purification of enzyme

As shown in Table 1), the specific activity of the purified protease from *P. chrysogenum* was found as 489 (units/mg prot/ml). Approximately 2.89 folds of purification were found by purification with 86.28% yield.

Table 1:Purification of amylase enzyme by ammonium sulfate precipitation

Purification step	Total enzyme Activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Recovery (%)
Culture supernatant	521	6.2	83.5	1	100
Ammonium sulphate precipitation	489	2.1	241.4	2.89	86.28

DISCUSSION

Alkaline protease can be derived from a variety of sources, including fungi, bacteria, and insects. Most alkalophilic microbes produce alkaline protease, however interest is concentrated on those that produce a large amount of enzyme. Several studies are being conducted on halophilic fungi, which produce industrially relevant enzymes. Protease production is carried out by fungi such as *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor* and *Humicola* [13]. The protease properties of *P. chrysogenum*, on the other hand, are mainly unexplored.

Agitation aids in the proper mixing of nutrients and is essential for microbial growth. Most research indicated that a speed of 150 rpm was optimal, however in this study, the production of protease was better in a shaking condition of 100 rpm as compared to static cultures. The effects of oxygen on enzyme synthesis in an aerobic fermentation process are related to changes in the metabolic pathway and metabolic fluxes [14]. According to Ducros *et al*. [15], the aerobic culture's respiration rate is influenced by dissolved oxygen. This is related to a change in cell metabolism caused by physiological changes [16]. Shaking conditions boosted protease synthesis in *A. flavus* LCJ253, which could be attributed to lower availability of dissolved oxygen with low mixing rates [17].

Carbon source is a key energy source that plays an important role in the improvement of bioprocessing of biochemical by supplying the suitable carbon sources. The influence of several carbon sources on protease production revealed that sucrose produced more than the other carbon sources used in the

experiment. Several studies have found that different carbon sources have distinct effects on the enzyme synthesis of various fungus. Other researchers have found increased alkaline protease production using other sugars such as lactose, maltose, sucrose, and fructose [18, 19], but not under salt stress. Okpara *et al*.[20] reported that the sucrose was the best carbon source for extracellular alkaline protease in *Aspergillus flavus*. Generally in addition to an energy source and trace elements, a suitable nitrogen source in adequate quantities is essential for the rapid growth of the fungus. In the present study, organic nitrogen substances were suitable for protease production. Accordingly, ammonium nitrate enhanced maximum protease production in *P. chrysogenum*. Similarly, Nascimento and Martins, [21] reported that the maximum protease activity (1.1U/mg Protein) was obtained when ammonium nitrate was used in the medium.

Enzyme production by the fungal culture is strongly dependent on the medium pH.It has an impact on the majority of enzymatic processes as well as the transport of various components through cell membranes [45, 46].In the present study, pH 9 (alkaline pH) favoured maximum protease production by *P. chrysogenum* of protease activity at 5% level. Similar results were reported by Benluvankar *et al*.[22] in *Penicillium*sp and Palanivel *et al*. [23] in *Aspergillus* strain KH17. Temperature is another critical parameter that has to be controlled for maximum cell growth and protease enzyme production. In this study, the production of alkaline protease from *P. chrysogenum* was optimized at a temperature range of 40˚C - 50˚C. Similar results were reported by Agrawal *et al*.[24] in *Penicillium* sp and Niyonzima and More, [25] in *Aspergillus terreus*.

High salinity disrupts normal protein function by changing protein solubility, binding, stability, and structure [26]. In comparison to conventional enzymes, salt-adapted enzymes from halophilic bacteria may keep protein structure and activity under saline conditions [27].In this investigation, at 4% of salt concentration, it was registered the maximum protease activity and minimum enzyme activity was obtained at 1% of salt concentration. Similarly, Okparaet al.(20) reported that the maximum extracellular alkaline and acid proteases were produced when the *Aspergillus* spp. were subjected to 4% salt stress.

40% ammonium sulphate saturation was found suitable to precipitate protein with the highest enzyme activity. Benluvankar *et al*.[22] reported that the 1.3-fold increase with 69% yield by ammonium sulphate precipitation of protease from *Penicillium sp.* and Muthulakshmi *et al*.[28] observed 2-fold purification with 66% of recovery by ammonium sulphate precipitation of protease from *Aspergillus flavus*.

CONCLUSION

The current work was shown that the halophilic fungus *P. chrysogenum* is an efficient protease producer. The optimization of protease production conditions under submerged fermentation will help in the large-scale synthesis of this promising enzyme. Future research on the molecular identification of *P. chrysogenum*, as well as the purification and characterization of protease from this organism will help to expand its uses in many biotechnological and environmental aspects.

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CITATION OF THIS ARTICLE

R. Devi, K. Revathi and N. Yogananth. Halotolerant protease enzyme production by *Penicillium chrysogenum* from Marakkanam Saltpans, Tamil Nadu, India. Bull. Env.Pharmacol. Life Sci., Spl Issue [1] 2022 : 1075-1080