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NEUTRAL PROTEASE PRODUCTION BY *RHIZOPUS OLIGOSPORUS* NCIM 1215 UNDER SOLID STATE FERMENTATION USING MIXED SUBSTRATES OF AGRO INDUSTRIAL RESIDUES

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ABSTRACT

Neutral protease production under solid state fermentation was carried out by using *Rhizopus oligosporus* NCIM 1215 and was screened with six different agro industrial residues for maximum production of protease. Among all the agro industrial residues evaluated, mixed substrates like coconut oil cake and wheat bran in the ratio of 1 : 4 supported the maximum protease production by *Rhizopus oligosporus* NCIM 1215. The physiological fermentation factors such as fermentation time, temperature, initial moisture content, initial pH, inoculum age and inoculum volume played a vital role in protease production and improvement in the yield was observed with the supplementation of carbon and nitrogen sources to the solid medium. Fermentation carried out with 4 days old culture and 55% initial moisture content at a temperature of 32°C, pH 7.0 for 5 days were found to be optimum for enzyme production by the fermenting organism. The best carbon source for the maximum production of neutral protease by this organism was lactose 1% (w/w) and the best organic and inorganic nitrogen sources were yeast extract (1% w/w) and ammonium chloride (1% w/w). It was observed that the fungus *Rhizopus oligosporus* NCIM 1215 is the potential organism for the production of neutral protease, which could find applications in the field of food and pharmaceutical industries.

Key words: Neutral protease, *Rhizopus oligosporus*, Coconut oil cake, Wheat bran, Solid state fermentation, Optimization.

INTRODUCTION

Proteases, also known as peptidyl/peptide hydrolases (EC 3.4.21-24 and 99), are industrially useful enzymes, which catalyze the hydrolysis of a peptide bond in a protein

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molecule[1]. They conduct highly selective and specific modification of proteins i.e. zymogenic form of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, processing and transport of secretory proteins across the membrane[2]. Proteases are used for various industrial applications, such as laundry detergents, leather preparation, food industry and protein recovery or solubilization and organic synthesis[2] and also used as active ingredients in the development of biopharmaceutical products like contact lens cleaner[3]. In dairy industries as milk-clotting agents (calf rennet composed mainly of chymosine and pepsin)[4] and as an agent for meat tenderization[5]. Proteases have also clinical and medical application (reduction of tissue inflammation)[6,7]. Proteases can be used in the treatment of diseases and conditions such as cancer and autoimmune diseases and also immunosuppressive agents. Furthermore, proteases may be used as vaccine adjuvants. Proteases are produced by a wide range of microorganisms including bacteria, fungus, yeast and also in mammalian tissues[8].

Different methods of fermentation technology can be applied for the production of neutral protease. Commercial production of neutral protease has been carried out using submerged fermentation (SmF) technique**.** But nowadays, solid state fermentation (SSF) has been emerging as a promising technology for the development of several, bioprocesses and products including the production of therapeutic enzymes on a large scale. Economically SSF offers many advantages, including superior volumetric productivity, use of simpler machinery, use of inexpensive substrates, simpler downstream processing and lower energy requirements[10,11] with submerged fermentation[12].

In this paper, the optimized production of neutral protease enzyme by *Rhizopus oligosporus* NCIM 1215 using mixed substrates like wheat bran and coconut oil cake under solid state fermentation was reported.

EXPERIMENTAL

Microorganism and inoculum preparation

The fungal strain *Rhizopus oligosporus* NCIM 1215 used in this study was procured from National Institute for Industrial Microorganisms (NCIM) National Chemical Laboratory (NCL) Pune, Maharashtra, India. The culture was routinely maintained on PDA slants and incubated for 7 days at 28^{o}C. Spore suspension was prepared from a freshly raised 7 day old culture of *R. oligosporus* on PDA slants by suspending in 10 mL of sterile distilled water.

Fermentation medium and culture conditions

Fermentation was carried out under solid state fermentation as by Paranthaman *et al*. The fermentation medium has the following composition in a 250 mL Erlenmeyer flask : 5 g of substrate was taken in 250 mL Erlenmeyer flask separately, moistened with salt solution [composition (% w/v) (g/100 mL) : ammonium nitrate 0.5, potassium dihydrogen orthophosphate 0.2, sodium chloride 0.1 and magnesium sulphate 0.1]. The flasks were autoclaved at 121^{o}C (15 lb) for 20 min, cooled to room temperature and then inoculated with 1 mL of the *R.oligosporus* spore suspension under aseptic conditions. The contents of the inoculated flasks were mixed thoroughly and incubated at the desired temperature in an incubator for desired length of period.

Optimization of the culture condition for neutral protease production

Various process parameters that enhance the yield of neutral protease by *R. oligosporus* NCIM 1215 under solid state fermentation were investigated. The impact of incubation time (1-10 days), incubation temperature (24-48^{o}C), initial moisture content of the substrate (35-105%), initial pH (2-10 adjusted with 1N HCl or 1N NaOH), inoculum age (1-10 days) and inoculum concentration (0.5-4 mL), were evaluated. Moreover, the effect of incorporation of additional carbon sources (glucose, maltose, sucrose, fructose, lactose, soluble starch and galactose at 1% w/w) and nitrogen sources (ammonium nitrate, ammonium sulphate, ammonium chloride, sodium nitrate, potassium nitrate, malt extract, yeast extract, urea, peptone and beef extract at 1% w/w) were also studied. All the experiments were conducted in triplicate and the mean values were reported.

Extraction of crude enzyme

A solution of tween 80 (0.1%) was added into the 100 mL of distilled water. 25 mL of water was added to the 5 g of fermented substrate and the substrate was homogenized on a rotary shaker at 180 rpm for 1 h and then filters the solution. The solids were removed by centrifuging the homogenate at 8000 rpm g at 4^{o}C for 15 min and the resultant clear supernatant was used for analytical studies.

Assay for neutral protease

To 200 µL of crude enzyme extract, 500 µL of casein (1%) and 300 µL of 0.2 mol/L phosphate buffer (pH 7.0) were added. The reaction mixture was incubated at 60^{o}C for 10 min and arrested by the addition of 1 mL of 10% TCA[14]. The reaction mixture was centrifuged at 8000 x g at 4^{o}C for 15 min and to the supernatant, 5 mL of 0.4 mol/L Na_2CO_3, 1 mL of 3-fold diluted Folin and Ciocalteau's phenol reagent were added. The resulting solution was incubated at room temperature for 30 min and the absorbance of the blue color

developed was read at 660 nm and its concentration was determined using tyrosine standard curve[15].

One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µg of tyrosine from substrate (casein) per minute under assay condition. Enzyme yield was expressed as the activity of neutral protease per gram dry substrate (U/gds).

RESULTS AND DISCUSSION

Screening of substrates

The selection of an ideal agro-industrial residues for the enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate material. The results in the present study indicated that protease production pattern varied with the type of agro-residue. This could be attributed to solid materials, dual role supply of nutrients to the microbial culture and anchorage for the growing cells. Six different substrates like black gram husk, rice bran, wheat bran, coconut oil cake, groundnut oil cake, soyabean powder were screened for the maximum production of protease (Fig. 1). Maximum activity was obtained for the coconut oil cake (122.79 U/gds).

Sandhya *et al.*[16] reported that wheat bran was the suitable substrate for protease synthesis by A. oryzae NRRL 1808.

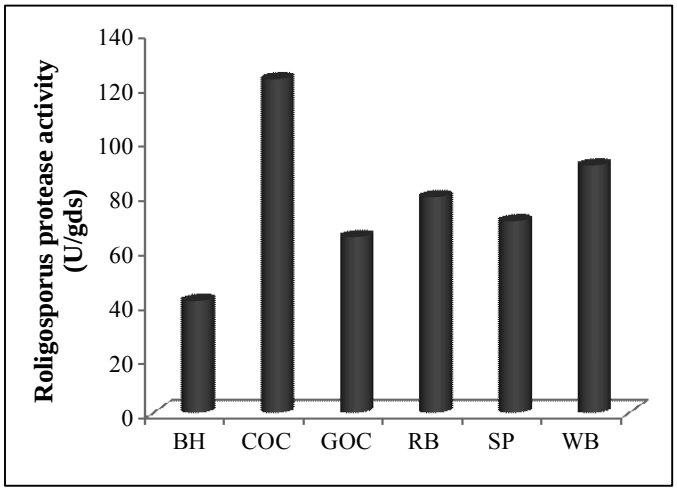

Fig. 1: Screening of substrates for the maximum protease production

BH = Black gram husk	COC = Coconut oil cake
GOC = Groundnut oil cake	RB = Rice bran
SP = Soyabean powder	WB = Wheat bran

Screening of mixed substrates

Out of the following substrates, maximum activity was achieved by using coconut oil cake (122.79 U/gds) followed by wheat bran (91.17 U/gds). So these two substrates were taken in different ratio's like (1 : 1, 1 : 4, 2 : 3, 3 : 2, 4 : 1) to achieve maximum activity of protease enzyme. Out of all the different ratios, coconut oil cake and wheat bran in the ratio of 1 : 4 gave maximum activity of (130.617 U/gds) neutral protease. So by using this ratio the experiment is further carried out (Fig. 2).

Chutmanop *et al.*[17] reported that substrate that had a wheat bran to rice bran ratio of 0.33 by dry weight was best for producing protease.

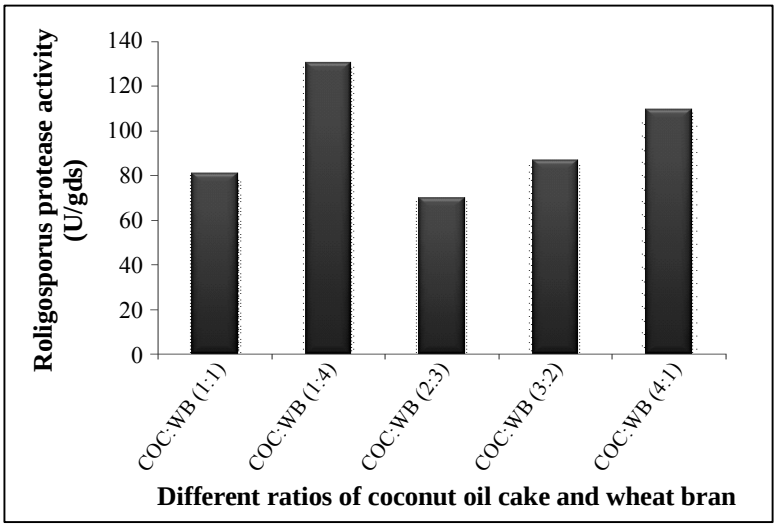

Fig. 2: Screening of mixed substrates for the maximum protease production

Effect of fermentation time on protease production

The production profile of neutral protease was studied by conducting the fermentation using *Rhizopus oligosporus* NCIM 1215 for different time intervals (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days). The enzyme production was gradually increased with the passage of time and highest enzyme activity (136.7647 U/gds) was obtained after 5 days of incubation (Fig. 3). A gradual decrease in enzyme units was observed with increase

incubation period clearly suggesting the enzyme role as a primary metabolite, being produced in the lag phase of the growth of fungus for utilization of nutrients (proteins) present in the solid substrate. The subsequent decrease in the enzyme units could probably be due to inactivation of the enzyme by other constituent proteases.

Radha *et al.*[18] reported that the highest protease activity was obtained after 120 hrs of incubation for culture medium containing molasses and cheese whey as substrates.

Shivakumar[19] reported that the maximum protease activity was obtained after 120 hrs of incubation for medium with wheat bran and gelatin 1% w/v as substrates.

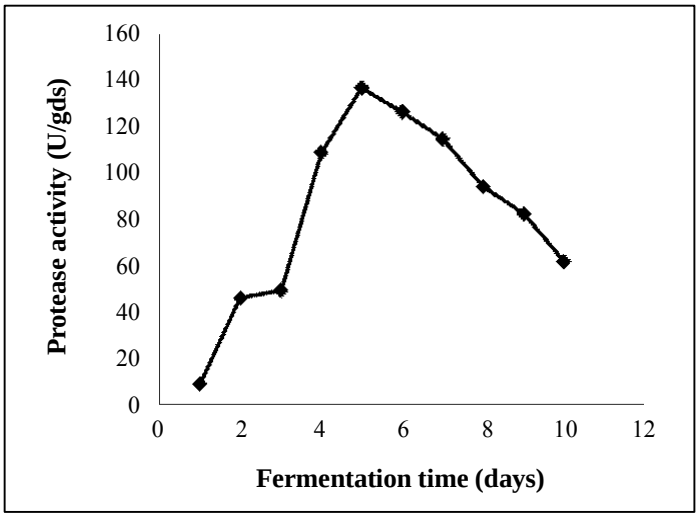

Fig. 3: Effect of fermentation time on neutral protease production by *Rhizopus oligosporus* NCIM 1215 using mixed substrates like coconut oil cake and wheat bran

Effect of fermentation temperature on protease production

The inoculated flasks were incubated at different temperatures to determine the optimum fermentation temperature for neutral protease production. The enzyme production was carried out by *R.oligosporus* at 24-48^{o}C temperature range. The optimum incubation temperature for the production of protease (141.1764 U/gds) was found at 32^{o}C **(**Fig. 4).

The enzyme production was reduced, when the incubation temperature was increased above 32°C. Higher temperature is found to have some adverse effects on metabolic activities of microorganism and cause inhibition of the growth of the fungus. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure.

In the work of Tunga *et al.*[20] the maximum production of protease by *Rhizopus oryzae* was obtained at temperature 32°C. Abdul Rauf *et al*.[21] reported that incubation temperature 35°C is the optimum for protease production using sunflower meal as a substrate.

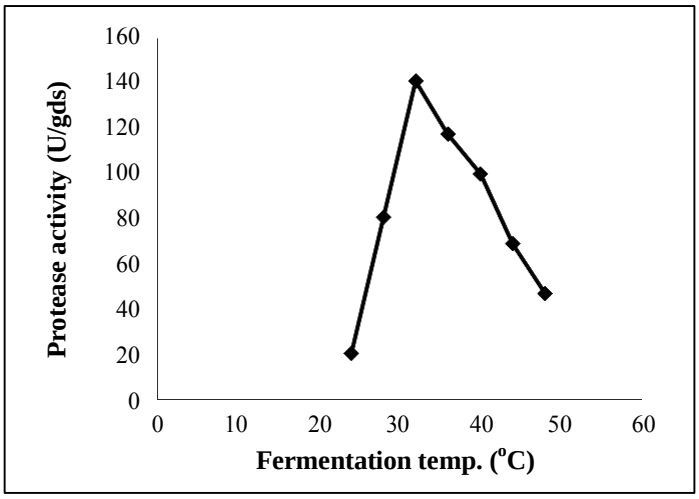

Fig. 4: Effect of fermentation temperature on neutral protease production by *Rhizopus oligosporus* NCIM 1215 using mixed substrates like coconut oil cake and wheat bran

Effect of initial moisture content

The optimum initial moisture content for neutral protease production was determined by adjusting the initial moisture content of the fermentation substrate to varying levels of 35-105%. Initial moisture content is a crucial factor affecting the formation of products through SSF. A moisture level of 55% was found to be optimum for neutral protease production (152.9411 U/gds) (Fig. 5).

A further increase in the initial moisture content beyond 55% resulted in a significant reduction in the enzyme production. Increase in moisture level is believed to

reduce the porosity of the substrate, thus limiting oxygen transfer, while lower moisture content causes reduction in solubility of nutrients of substrate and lower degree of swelling. Hence, an optimal level of moisture is required for maximum enzyme productivity.

Moisture content of 50% facilitated neutral protease production using *Aspergillus oryzae* (Ozykat-1), on wheat bran to rice bran ratio of 0.33 was reported by Chutmanop *et al*.[17]

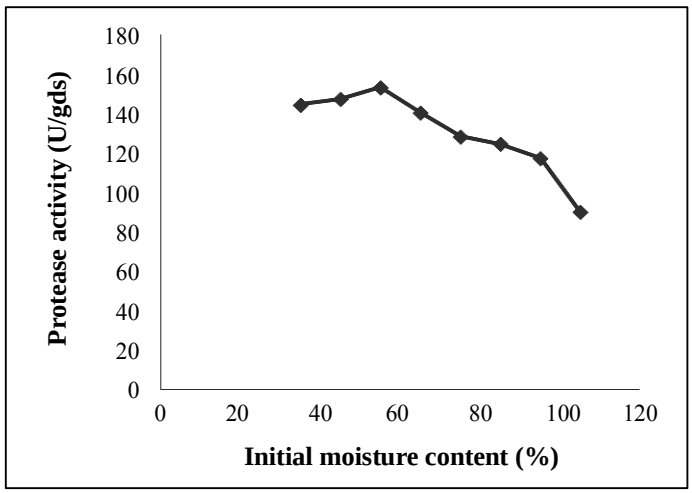

Fig. 5: Effect of initial moisture content on neutral protease production by *Rhizopus oligosporus* NCIM 1215 using mixed substrates like coconut oil cake and wheat bran

Effect of initial pH on protease production

The optimum pH for neutral protease production was determined by conducting the fermentation at different pH of medium namely 3-10 pH. The maximum enzyme production of 155.8 (U/gds) was obtained at neutral (7.0) pH (Fig. 6).

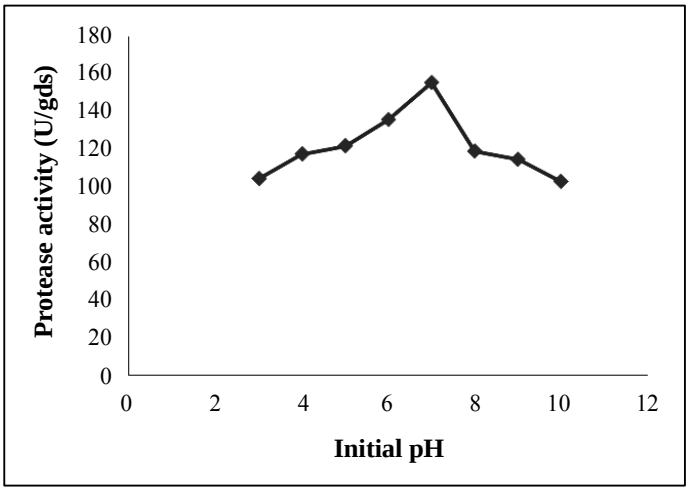

Fig. 6: Effect of initial pH on neutral protease production by *Rhizopus oligosporus* NCIM 1215 using mixed substrates like coconut oil cake and wheat bran

Protease production by microbial strains depends on the extra cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support cell growth and product production. This may be attributed to the balance of ionic strength of plasma membrane. A notable decline in the enzyme productivity occurred at both; higher and lower pH values.

Similar results were also reported by Paranthaman *et al.*[13], where the maximum production of neutral protease from rice mill waste by *A. niger* was obtained at pH 7.0.

Effect of inoculum age on protease production

The effect of inoculum age on protease production was studied by conducting the fermentation with different inoculum ages. The substrate was inoculated with 1-day old culture to 10-days old culture in different flasks. The substrate was incubated at 32^{o}C for 5 days. After the completion of fermentation, the enzyme was extracted and analyzed for the protease activity. The four days old culture gave maximum production of protease (157.352 U/gds) (Fig. 7).

Ikasari and Mitchell[22] reported that the 5-day old inoculum gave best protease yield with *Rhizopus oligosporus* ACM 145F.

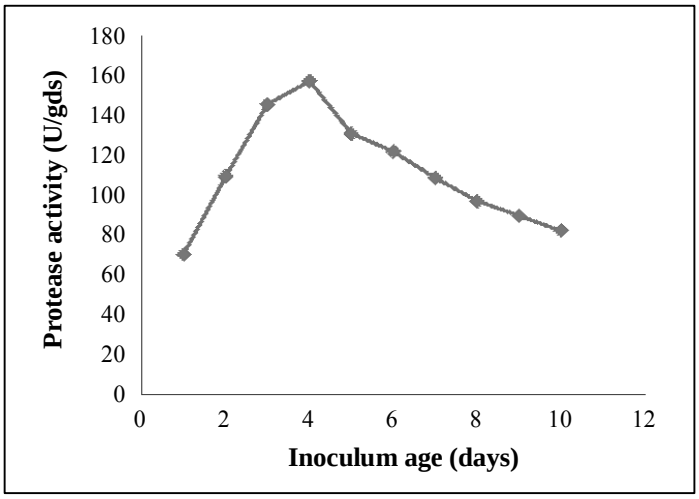

Fig. 7: Effect of inoculum age on neutral protease production by *Rhizopus oligosporus* NCIM 1215 using mixed substrates like coconut oil cake and wheat bran

Effect of inoculum volume

Fermentation was carried out with different inoculum volumes varying from 0.5-4 mL (1 x 10^6 spores/mL) for a period of 5 days to study its effect on the production of neutral protease. Maximum neutral protease production of 161.76 U/gds was obtained with 1 mL of 4 day old culture of *R. oligosporus.* With further increase in inoculum volume; there was a gradual decrease in the enzyme production because much increase in inoculum volume caused overcrowding of spore that decreased the enzyme activity (Fig. 8).

Abdul Rauf *et al*.[21] reported maximum enzyme production with 1 mL (1 x 10^6 spores/mL) inoculum size.

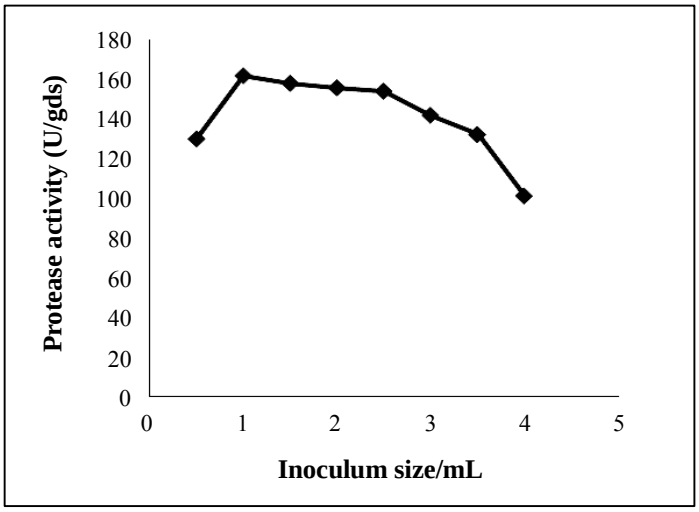

Fig. 8: Effect of inoculum volume on neutral protease production by *Rhizopus oligosporus* NCIM 1215 using mixed substrates like coconut oil cake and wheat bran

Effect of carbon supplements on protease production

Influence of various carbon supplements on enzyme production was studied by adding various sugars namely fructose, D-galactose, D-glucose, lactose, maltose, soluble starch and sucrose at 1% (w/w) to the fermentation media. Of the seven different carbon sources used as enrichment, lactose was a good carbon source, which gave maximum protease activity (164.70 U/gds) (Fig. 9).

Negi and Banerjee[23], reported that lactose was the best carbon supplement for the maximum production of protease. El-Safey and Abdul-Raouf[24] have also reported that lactose was the best carbon supplement for the maximum production of protease.

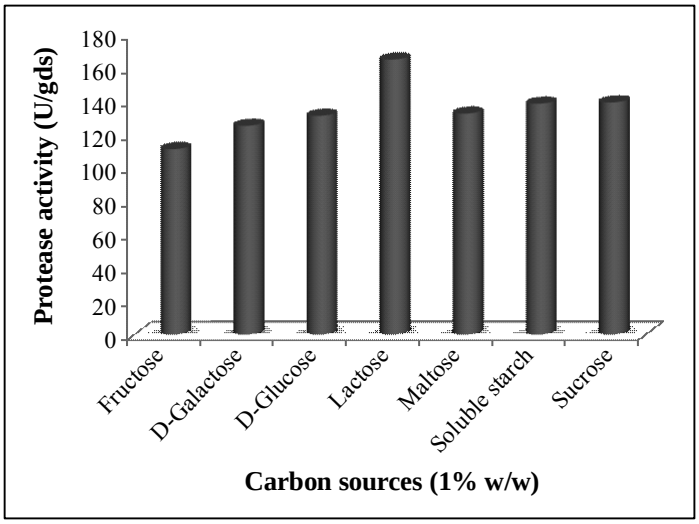

Fig. 9: Effect of carbon supplement (1% w/w) on neutral protease production by *Rhizopus oligosporus* NCIM 1215 using mixed substrates like coconut oil cake and wheat bran

Effect of organic nitrogen supplements on protease production

Various organic nitrogen supplements namely beef extract, malt extract, peptone, urea and yeast extract at a concentration of 1% (w/w) were added to the fermentation media to study its effect on enzyme production. The flasks containing substrates were inoculated and incubated for 5 days. Organic nitrogen supplement yeast extract was enhanced the protease production (169.117 U/gds) (Fig. 10). Certain nitrogenous salts tend to decrease the pH of the culture medium and had the adverse effect on enzyme production, although they supported the growth of the organism.

Nitrogen sources have a great effect for microbial growth and in the production of extra cellular enzymes[25].

Prakasham *et al.*[26] and Murthy and Naidu[27] reported that yeast extract was the best organic nitrogen supplement for the maximum production of protease.

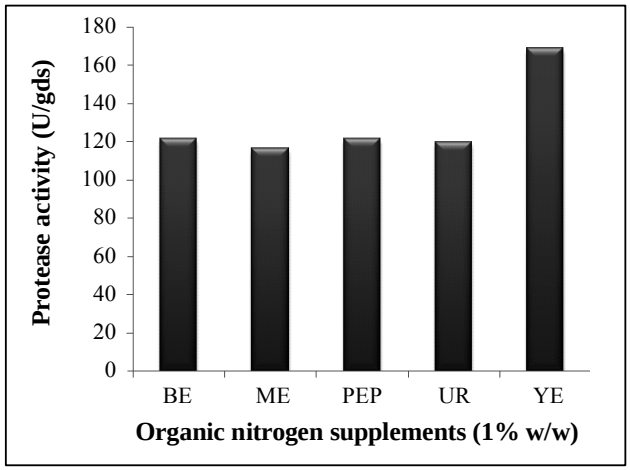

Fig. 10: Effect of organic nitrogen supplements (1% w/w) on the production of neutral protease by *Rhizopus oligosporus* NCIM 1215 using mixed substrates like coconut oil cake and wheat bran

Effect of inorganic nitrogen supplements on protease production

Various inorganic nitrogen supplements namely ammonium nitrate (NH_4NO_3), ammonium sulphate $(NH_4)_2SO_4$, ammonium chloride (NH_4Cl), potassium nitrate (KNO_3) and sodium nitrate ($NaNO_3$) at a concentration of 1% (w/w) were added to the fermentation media to study its effect on enzyme production. The inorganic nitrogen supplement ammonium chloride enhanced the protease production (171.42U/gds) (Fig. 11).

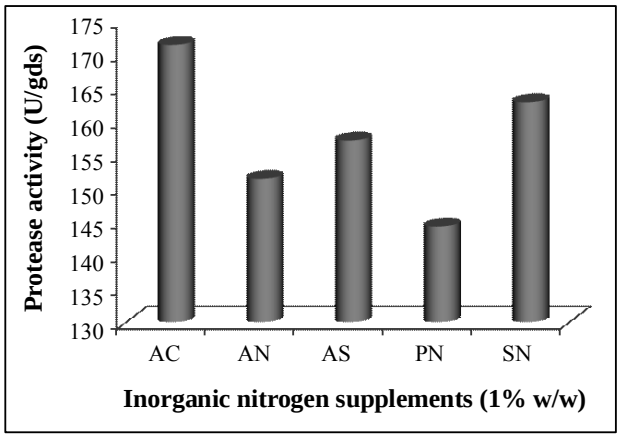

Fig. 11: Effect of inorganic nitrogen supplements on the production of neutral protease by *Rhizopus oliosporus* NCIM 1215 using mixed substrates like coconut oil cake and wheat bran

Mehrotra *et al*.[28] reported that ammonium chloride was the best inorganic nitrogen supplement for the maximum production of protease.

The results obtained in the study shows that *Rhizopus oligosporus* NCIM 1215 is more effective for the production of neutral protease using the mixed substrates of coconut oil cake and wheat bran in the ratio of 1 : 4. It is observed that the mixed substrates in various ratios were found more effective than the single substrates used in the production of neutral protease. Hence the present study was carried out using mixed substrates. These agro-industrial residues coconut oil cake and wheat bran are cheaper and commercially available. The neutral protease obtained from *Rhizopus oligosporus* NCIM1215 could find applications in food and pharmaceutical industries.

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