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Red gram husk: A potent substrate for production of Protease by *Bacillus cereus* in Solid-State Fermentation

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Abstract: Agro residue Red gram husk were used as substrate/solid support for production of protease under solid-state fermentation using *Bacillus cereus*. Red gram husk supported good bacterial growth and enzyme production (protease 262 U/g of substrate). This works investigates the impact of various physical and chemical fermentation factors. Maximum protease production was observed at a growth of 48 hrs, a substrate pH of 9.0, 50 % substrate moisture, temperature of 40ºC and 7.5g of substrate concentration . Enrichment with Fructose as carbon source increased protease production. Nitrogen supplementation with Beef extract also enhanced the enzyme yield reaching 258 U protease activity per gram of Red gram husk. The enzyme production was found to be associated with the growth of the bacterial culture. The results presented in this work show the potential for utilization of Agro residue by solid-state fermentation for the production of industrial enzymes.
Keywords: protease, Red gram husk, *Bacillus cereus*, Optimization, SSF.

INTRODUCTION

Proteases, also known as proteinases or proteolytic (protein-digesting) enzymes that are mainly classified on the basis of their pH optimum as acidic, neutral, and alkaline proteases, are large group of enzymes. Proteases are involved in digesting long protein chains into short fragments, splitting the peptide bonds that link amino residues. Proteases are used throughout an organism for various metabolic processes. Proteases determine the lifetime of other proteins playing important physiological role like hormones, antibodies, or other enzymes. This is one of the fastest "switching on" and "switching off" regulatory mechanisms in the physiology of an organism [1].

Among all the different commercial enzymes, microbial protease in particular, represents about 60% of all the industrial enzyme's sales in the world due to their applications in several industrial sectors like in the detergent, food, pharmaceuticals, chemicals, leather, paper and pulp and silk industries[1]. The most of enzymes employed in industrial production of foodstuffs, biochemical reagents and other important compounds originate mainly from bacteria, especially from varieties of genus Bacillus[2]. The amount of protease produced varies greatly with strains and media used Microbial proteases are gaining more importance than conventional chemicals that cleave peptides because of the cheaper production cost and use of renewable resources. Microbial proteases can be produced from bacteria, fungi and yeast using many processes like solid-state fermentation, submerged fermentation [3 4 5]

Bacterial cultures, in general, were considered non-suitable for SSF due to high-water activity requirement. However, experience has shown that

bacterial cultures can be well managed and manipulated for SSF processes.[6] [7] Pandey has conclusively demonstrated that bacterial SSF works efficiently for the production of alkaline protease, a-amylase and inulinase by Pseudomonas sp., Bacillus sp. and staphylococcus sp.[8] [6] [9]

At present, the overall cost of enzyme production is very high (due to high cost of substrates and mediums used) and therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable from the commercial point of view.

To achieve these goals, during the recent years, efforts have been directed to explore the means to reduce the protease production costs through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural byproducts as substrate(s) for protease production.[10] [9] Protein and enzyme production using solid waste as the substrate has been demonstrated as a viable technique for by-product recovery.[11] Application of agro industrial residues as substrates is certainly economical and it also reduces environmental pollution. Several naturally occurring agricultural byproducts such as wheat bran, coconut oil cake, groundnut oil cake, rice bran, wheat and paddy straw, sugar beet pulp, fruits pulps and peels, corn cobs, saw dust, maize bran, rice husk, soy hull, sago hampas, grape marc, coconut coir pith, banana waste, tea waste, cassava waste, aspen pulp, sweet sorghum pulp, apple pomace, peanut meal, cassava flour, wheat flour, corn flour, steamed rice, stream pre-treated willow, starch etc. could be used in one or the other industrial bioprocess for the production of value added products through SSF[12].

There are several reports describing use of agro-industrial residues for the production of alkaline protease, e.g. nug meal and *bacillus* sp. AR009.[13] pigeon pea and *bacillus* sp. JB-99[14] wheat bran and *Rhizopus oryzae* [15].

The primary objective of this work was to carried out Solid state fermentation using Red gram husk as a substrate for Protease production and optimize its process parameters, the effect of incubation time, pH of the medium, % of substrate moisture, incubation temperature, substrate concentration and Supplementation of co-carbon and co-nitrogen sources. No attempt has been made elsewhere to use Red gram husk as a substrate for protease enzyme production.

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MATERIALS AND METHODS

Bacterial strain

Bacterial strain used in this work is well preserved in the laboratory. Bacterial strain *Bacillus cereus* was a stock of the Microbial Type Culture collection Centre (MTCC), Chandigarh, India. The strain was maintained on nutrient agar slants at 35+2◦C and pH 7.5. The medium composition (g/l) was comprised off the following: Beef extract 3.0; Yeast extract 2.0; Peptone 5.0; NaCl 5.0; Agar 15:pH 7.2. Cells were sub cultured at monthly intervals.

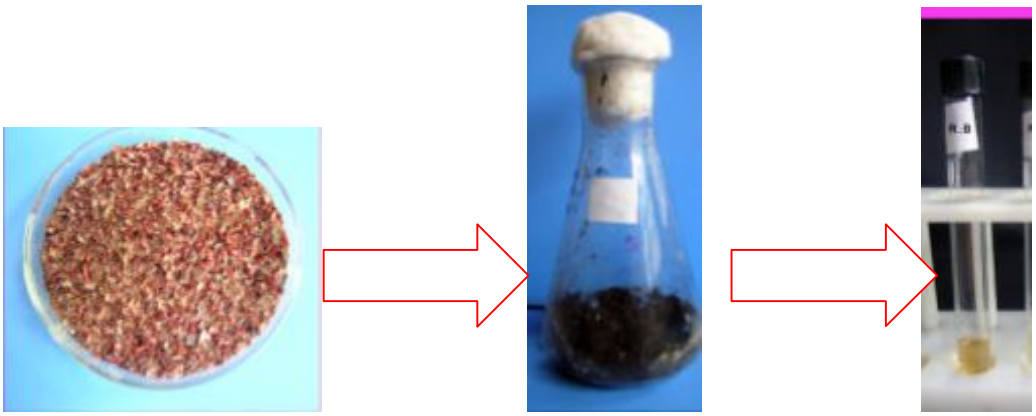
Selection of substrate

Black gram is split in a traditional stone grinder used for splitting and grinding grains. Cleaned, split black gram is soaked in water overnight (16 hours) and the husk is removed by hand washing 2 to 3 times in water. The removed husk is used as substrate and processed using USA standard sieve set Nos. 7, 10, 14, 18 and 50 to obtain mean particle size of 2.8-2.0; 2.0-1.4; 1.4-1.0 and 1.0-0.3 mm and stored till further use.

Solid-state fermentation process

Ten grams of rice bran was taken in a 250 ml Erlenmeyer flask and to this predetermined quantity of water was added, mixed thoroughly and autoclaved 121°C for 15 min. After cooling the flask to room temperature, the flasks were inoculated with 2ml of 24-h grown culture broth under sterile conditions. The contents of the flasks were well mixed with 50% relative humidity and incubated at 33±1ºC for 120 hrs. Fermentation was conducted under various experimental conditions

During the preliminary screening process, the experiments are carried out for 5 days and it was found that at the 48 hrs, the maximum production occurs. Hence experiments are carried out for 48 hrs.



Red gram husk **Protease production** **Crude protease**

Fig 1. Schematic Diagram of Protease production on Red gram husk by *Bacillus cereus*

Extraction of Protease

The enzyme was extracted according to the method described by Nagamine et al.,[16] . Fermented medium was mixed thoroughly with 50 mM glycine–NaOH buffer, pH 11 for 30 min and the extract was separated by squeezing through a cloth. This process was repeated three times and extracts were pooled together and then centrifuged. The supernatant was used as enzyme source for protease assay.

Determination of protease activity

Protease activity was determined using modified Auson–Hagihara method[17] . In this 1 ml of the enzyme solution was added to 1 ml casein solution (1%, w/v casein solution prepared in 50 mM glycine–NaOH buffer, pH 11) and incubated at 70ºC for 20 min. The reaction was terminated by adding 4 ml of 10% trichloroacetic acid and the contents were filtered through a Whatman No. 1 filter paper. The filtrate absorbance was read at 280 nm using UV–Visible spectrophotometer and the protease activity was calculated using tyrosine standard curve. One unit of alkaline protease activity was defined as 1 µg of tyrosine liberated ml¯1 under the assay conditions

Optimization of various parameters

Red gram husk as a substrate were employed for optimization of process parameters. To investigate the influence of culture parameters on protease production, effects of various time periods (24, 36, 48, 72, 96 and 120 h), initial pH(5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0),initial moisture content of the substrate (50%, 100%, 200% and 300%), incubation temperature (30, 35, 40, 45and 50 ºC), substrate concentration (2.5,5.0,7.5, 10 and 12.5 g). Co-carbon sources (glucose, fructose, galactose, maltose, sucrose and lactose at 10.0%, w/w) and co-nitrogen sources (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, yeast extract, beef extract, casein and peptone at 1.0%, w/w) were studied. For initial moisture content, solid substrate was mixed with predetermined amount of water and 100% moisturization Was achieved by adding 1.0 ml water to 1.0 g substrate and vice versa[9].

Optimization of various parameters was carried out with "One at a time" strategy keeping all other variables constant except one. The variable under study was varied over a desired range in triplicates and optimal level was used for further investigation.

Estimation of total soluble protein

Protein concentration was determined by method of Lowry et al.[18] using bovine serum albumin as standard and was expressed as milligram protein per gram dry fermented substrate

RESULTS AND DISCUSSION

The selection of an ideal agro-biotech waste for enzyme production in a solid-state fermentation process depends upon several factors, mainly related with cost and availability of the substrate material. The primary objective of this work was to check the suitability of Red gram husk, as a substrate for growth

of *Bacillus cereus* and protease production, since a large amount of Red gram husk is generated as a by-product during processing of Red gram husk. This is to explore the meaningful utilization of this by-product. Pandey et al.,[8] has conclusively demonstrated that bacterial SSF works efficiently for the production of alkaline protease, Bacillus sp.[9]

Influence of incubation time on enzyme production

The incubation time is governed by characteristics of the culture and also based on growth rate and enzyme production. Fig.2 shows a gradual increase in enzyme production, maximum at 48 hrs (258 U/gds). The enzyme yield showed a gradual decrease on further extension of fermentation period. The decrease in enzyme yield after the optimum level may be because of denaturation or decomposition of protease due to interaction with other components in the medium. These results are in accordance with observations made by B. Johnvesly[19].

Influence of initial pH on enzyme production

The pH of the fermentation media may change during fermentation because the substrates employed in SSF usually have the least buffering. Some samples from the fermented.

Mass were aseptically withdrawn, homogenized and pH was checked. The pH is another important factor which affects the growth and enzyme production during solid-state fermentation[20]. Fig. 3 shows that a maximum protease production was observed at pH 9.0 (260 U/g substrate), and then remained more or less constant in the pH range .0-11.0 This suggested that the bacterial strain was alkalophilic in nature.

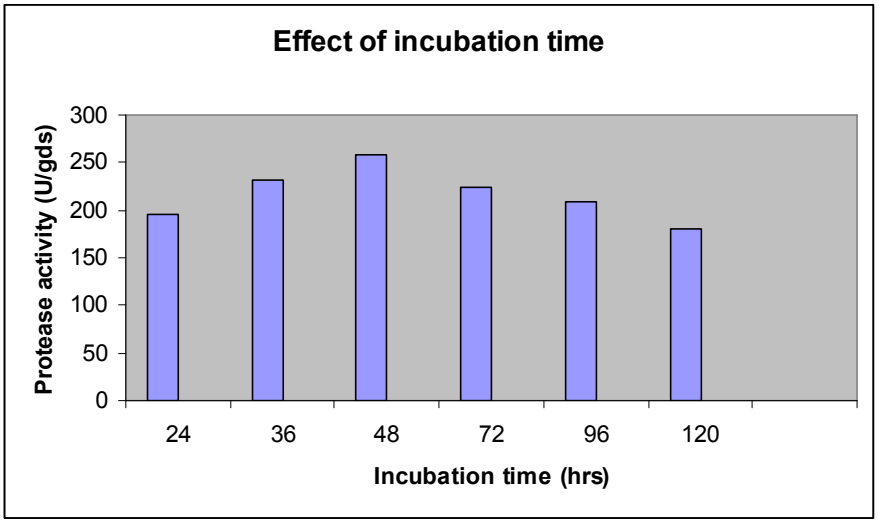


Fig.2 Effect of incubation time on protease production by *B. cereus*

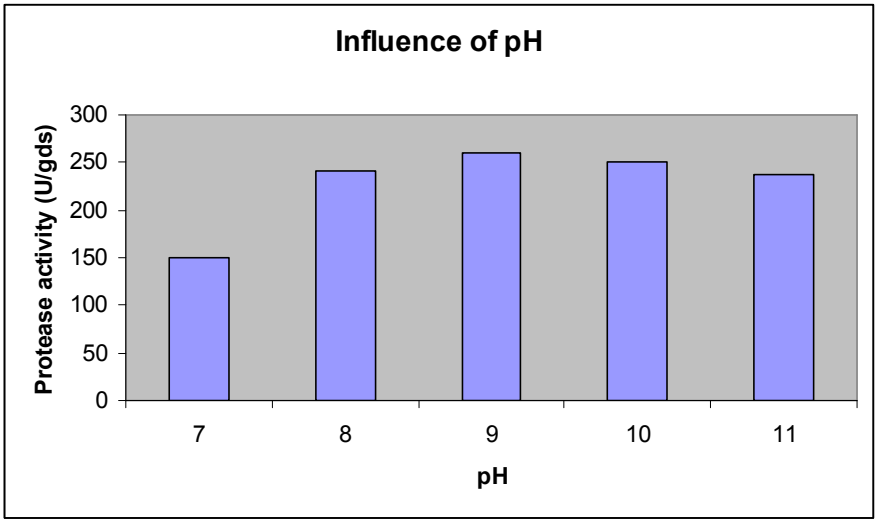


Fig.3 Effect of initial pH on protease production by *B. cereus*

Effect of initial moisture content of the substrate

The moisture content is a critical factor in solid-state fermentation. Its importance for microbial growth and thereby enzyme production has been well established. To check the influence of moisture on protease activity during SSF, Red gram husk was moistened with different amounts of distilled water (30%, 50%, 65%, 75% and 80%) prior to fermentation. Results summarized in Fig.4 shows that 50% of moisture was the optimal giving maximum amount of protease production (248U/g of substrate). An increase and decrease in the moisture content significantly affected enzyme production. The moisture level in SSF has a great impact on the physical properties of the substrate[21]. An increase in moisture content causes a decrease in the porosity of the substrate, thereby decreasing the gas exchange. Low moisture content leads to sub-optimal growth and a lower degree of substrate swelling which also decreases enzyme production.

Influence of incubation temperature on enzyme production

The influence of temperature on protease production is related to the growth of the organism. The results from fig.5 show that the enzyme yield was maximum (240 U/gds) in the control at 40ºC .It was interesting to note that at 30 ºC , the yield was 180 U/gds and at 50 it was 208 U/gds. It has also been reported that the metabolic heat generated during microbial cultivation in SSF exerts harmful effects on the microbial activity and thus the initial set temperature is vital.

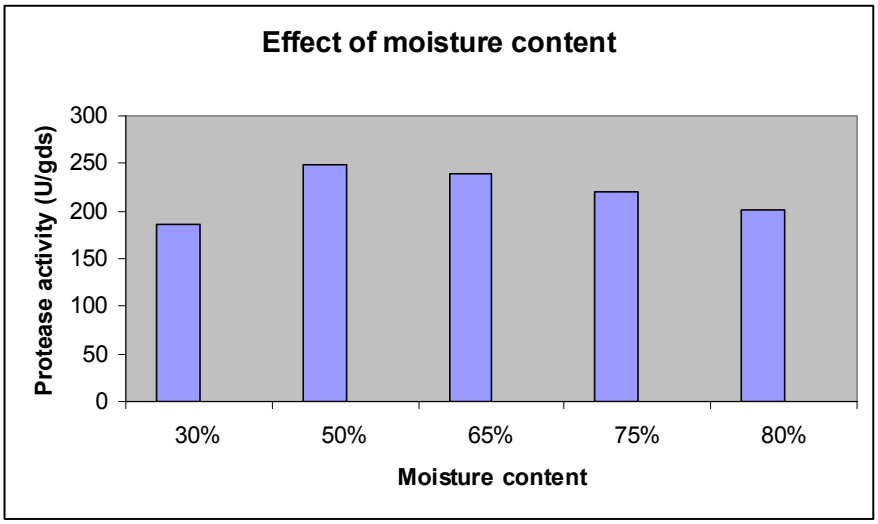


Fig.4 Effect of initial moisture content on protease production by *B. cereus*

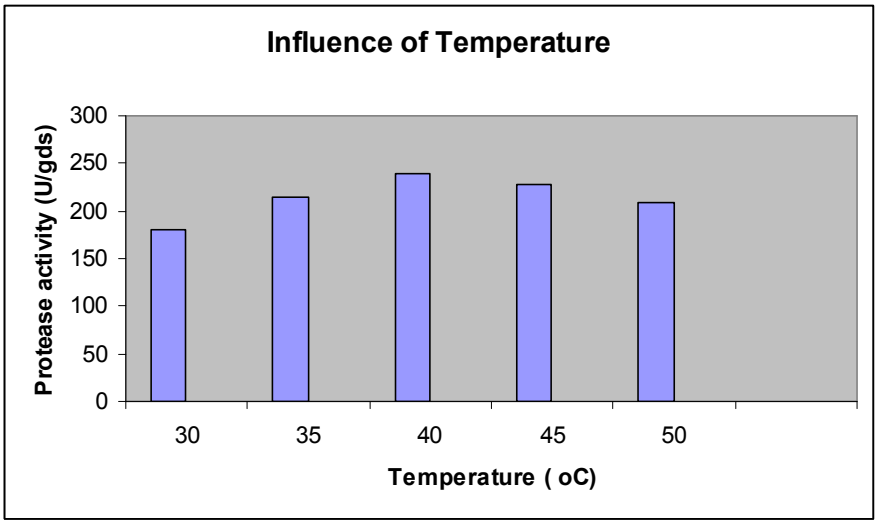


Fig.5.Effect of incubation temperature on protease production by *B. cereus*

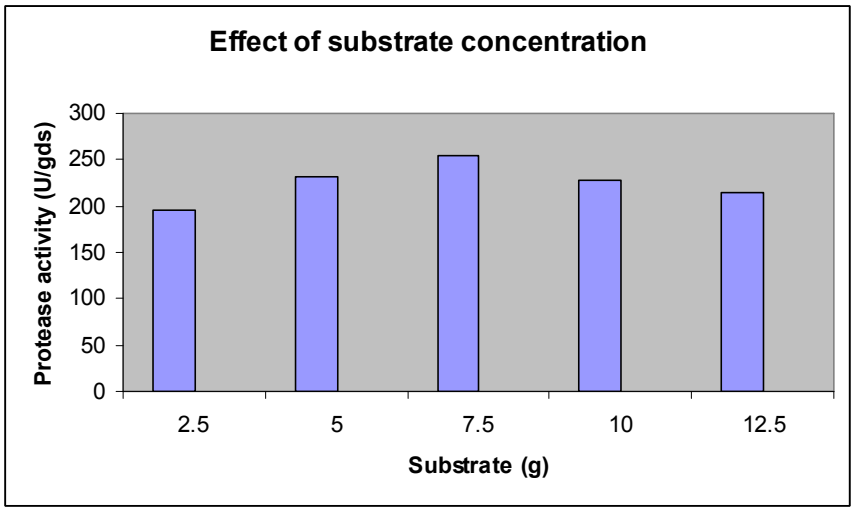


Fig.6.Effect of substrate concentration on protease production by *B. cereus*

Influence of substrate concentration on enzyme production

To determine the effect of substrate concentration on protease production , the production medium of different concentration i.e., 2.5,5.0,7.5, 10and 12.5g were prepared at pH 9.0 in 250 ml flasks was inoculated and incubated at 40ºC in a incubator for 48hrs. The results in the fig.6 indicated that the concentration from 2.5g to 7.5g the enzyme production increased the maximum production showed was 254 U/g of substrate. After 7.5g the enzyme production decreased with the increase in substrate concentration.

Effect of different carbon sources

Several carbon sources such as glucose, fructose, galactose, maltose, sucrose and lactose at 10.0%, w/w ratio were supplemented to solid medium. As depicted in Fig. 7, optimum protease production was observed when SSF was carried out with fructose (10%, w/w) as co-carbon source The flask were inoculated and incubated at 40ºC in a incubator for 28 hrs. In order to know optimum concentration of carbon source enzyme production was investigated by supplementation of different fructose concentration (1.0-10.0%).Maximum production (264U/g substrate) occurred with 5.0% fructose concentration. Further increase in this carbon source adversely affected protease production in this *Bacillus* strain under solid-stats fermentation environment. These results were in accordance with reported protease production in the presence of different sugars[9].

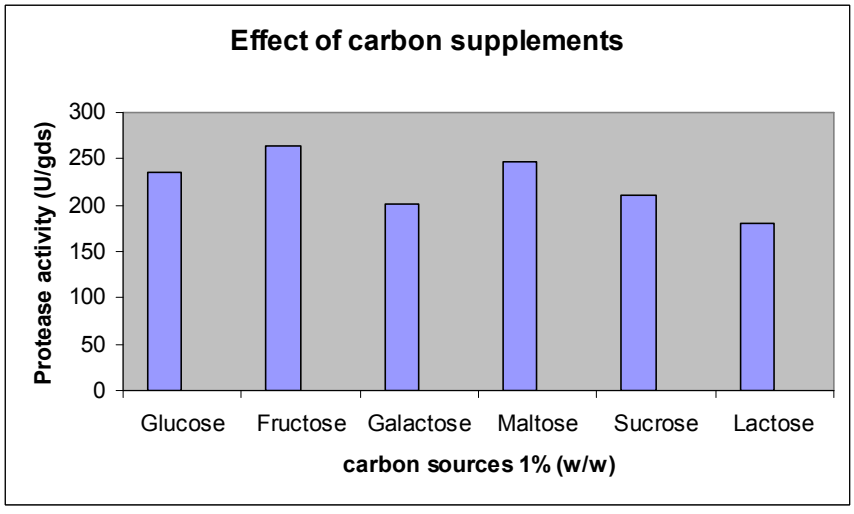


Fig.7 Effect of carbon sources on protease production by *B. cereus*

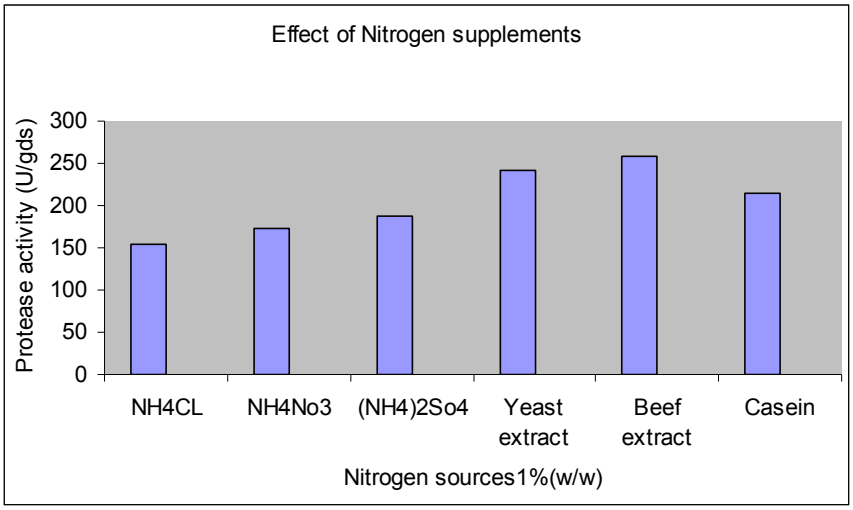


Fig.8 Effect of nitrogen sources on protease production by *B. cereus*

Role of different nitrogen sources

Among the tested nitrogen compounds, supplementation of 0.1% (w/w) beef extract followed by yeast extract lead to optimum protease production by *B. cereus* strain in SSF

According to Fig.8, maximum protease production was recorded in culture containing beef extract as nitrogen source reached to 248 U/g substrate, followed by yeast extract Similar observation were noticed in case of protease production by different microbial species[22].To optimize the beef extract concentration as nitrogen source for the production of protease, experiments were conducted with increase in beef extract supplementation in the medium. The results indicated an improvement in protease production with increase in beef extract concentration in the medium.

CONCLUSION

The present investigation relates to a simple, novel, low-cost process for the high-level production of alkaline protease from *B. cereus* by using a Red gram husk. Although a number of solid substrates have been used for the production of bacterial proteases, Red gram husk have never been used for protease production either in SSF or SmF systems. The major advantage of the present method of protease production is the commercial utilization of Red gram husk. Alkaline protease production by bacillus sp. strain under solid-state fermentation was influenced by physiological and chemical nature of the Red gram husk and associated with growth of the microbial strain. The optimum conditions for the maximum protease production (262 U/gds) using cassava waste are as follows: incubation time 48 hrs, initial pH 9,initial moisture content 50%, incubation temperature40 ºC, and substrate concentration 7.5 g (% w/w). The choice of the nitrogen and carbon sources has a major influence on the yield of protease. Present observation as well as findings from other laboratories suggested that different bacteria have different preferences for either organic or inorganic nitrogen for growth and protease production, although complex nitrogen sources are usually used for alkaline protease production [9]. Out of all the carbon sources which include (glucose, fructose, galactose, maltose, sucrose and lactose at 10.0%, w/w) and co-nitrogen sources (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, yeast extract, beef extract, casein and peptone at 1.0%, w/w) fructose has been found to be the best carbon supplement next is maltose and then glucose and sucrose. Out of all the nitrogen sources beef extract has been found to be the best nitrogen supplement next to yeast extract and then casein followed by peptone and $(NH_4)_2SO_4$. After considering all the optimized conditions of all physico-chemical parameters considered the total yield of protease enzyme has been found to be 262 U/gds. The enzyme production in this range from this vastly available by-product is significant. Other commonly employed agro-wastes have been reported to produce protease activity in the similar range; viz. 266 U/g[6], 1210 U/g[7], 10,772 U/g[23].However, with the use of still sophisticated equipment with all the required controlling parameters could further enhance the production of protease.

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