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Optimization of culture conditions for extracellular protease production from yeast isolate Y46

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ABSTRACT

In the present study, ten isolates from fermented dairy products were screened for protease production. Out of ten isolates, two isolates were found to produce protease enzyme and yeast isolate Y-46 was selected for further study. Protease production by yeast isolate Y-46 was studied in different fermentation conditions. The effects of various medium components and physicochemical conditions including production profile, inoculum size, inoculum age, carbon source, nitrogen source, pH and temperature on extracellular protease production were studied. The maximum protease production was observed after 24 h of production time with 3.0 %, v/v inoculum size with optimized fermentation medium containing galactose (2 %, w/v), peptone (2 %, w/v) and yeast extract (1 %, w/v). The optimum pH and temperature for maximum protease production were found to be 8.0 and 30 ºC, respectively. The classical "one-factor-at-a time" approach increased protease activity of Y-46 yeast isolate from 0.033 U/ml to 0.42 U/ml, indicating 12.73 fold enhancements after optimization.

Keywords: Dairy products, extracellular, optimization, protease, yeast.

INTRODUCTION

Proteases are the enzymes that have the catalytic ability to hydrolyze the peptide bond in a protein, causing it to disintegrate into polypeptides and amino acids. These enzymes are derived from plants, animals, and microbes. Peptide bond cleavage can be specific, resulting in selected protein cleavage for post-translational modification and processing, or it can be general, resulting in the breakdown of protein substrates into their constituent amino acids [1]. According to the type of functional group at the active site, proteases can be classified as serine protease, aspartic protease, cysteine protease, or metalloprotease [2].

Proteolytic enzymes are important because to the numerous medical treatments that utilize them and due to their significance as reagents in laboratory, clinical, and industrial processes. One of the three main categories of industrial enzymes, proteases account for around 60% of all enzyme sales globally [3]. For a variety of reasons, microbial sources of proteases are preferred to those from plants and animals. It is known that a wide range of microbes, including bacteria, fungi, yeast, and actinomycetes, produce these enzymes [4].

Despite the fact that bacteria from the genus Bacillus are the source of the majority of commercially available proteases, fungus has a greater range of proteases than bacteria. Researchers are interested in fungus because they can grow on inexpensive substrates and produce a lot of enzymes into the culture medium, which could simplify downstream processing [5].

A variety of parameters, including temperature, pH, carbon and nitrogen source, affected the production and yield of the enzyme [6, 7]. In the present study, protease producing yeast were isolated from dairy products (*dahi, lassi*) and screened. The enzyme was then produced in shake flask and various production parameters were optimized.

MATERIAL AND METHODS

Chemicals
All of the chemicals employed in the present study were of the highest quality analytical grade and were purchased from Sigma Aldrich (U.S.A.) and Hi-Media (Mumbai, India).

Sample collection
The samples of dairy products (*dahi, lassi*) were collected from Shimla, Himachal Pradesh. The samples were collected in sterile vials and stored at 4ºC till further processing.

Isolation of yeast
After homogenizing one gram of the sample with nine ml of saline, serial dilutions ranging from 10^{-1} to 10^{-10} were made. An aliquot of 0.1 ml of each dilution was spread on YM Agar (Yeast Malt Agar) supplemented with ampicillin (0.05 g/L) and acidified with 1N HCl to pH 5.0. The inoculated plates were incubated for 24 h at 30 ºC.

Screening by plate assay method
Isolates were grown in YPD broth for 24 h and centrifuged at 10,000 x g for 5 min at 4 ºC and supernatant was collected. Wells were made on skimmed milk medium (skimmed milk powder 10 % w/v) and filled with 50 µl cell free supernatant of 24 h old cultures. Plates were kept for 2 h at room temperature for diffusion of supernatant and incubated for 24 h at 30 ºC [8]. The diameters of zone of proteolytic activity around the wells were measured and clear zone of 1mm or more was considered as positive for proteolytic activity.

Protease production
The yeast isolate was inoculated in 50 ml of production broth containing galactose 2 % w/v, peptone 2 % w/v and yeast extract 1 % w/v. After 24 h of incubation, 1.5 ml of inoculum was transferred to 50ml of production medium (pH 8) and incubated at 30 °C for 24 h in an incubator shaker (150 rpm), and then centrifuged at 10,000 rpm for 10 min at 4 °C and the cells were discarded and the supernatant was assayed for the extracellular protease activity.

Measurement of enzyme activity
Protease activity in the crude enzyme extract was determined according to the method of Manachini et al. [9] using casein as substrate. The reaction was performed in 1 ml reaction mixture containing 700 µl of 50 mM tris-HCl buffer pH 7.0, 50 µl of supernatant and 250 µl of casein. In control reaction supernatant and buffer was added. The reaction was carried out in the test tube at 30 °C in shaking water bath for 30 min and the reaction was stopped by adding 1 ml of 5 % TCA (Trichloroacetic acid). The substrate was then added to the control tube. The reaction mixture was centrifuged at 10,000 rpm for 5 min and the optical density (OD) of the supernatant was taken at 275 nm. The standard curve was prepared with tyrosine (10-100 µg/ml). One unit of the enzyme activity is defined as the amount of enzyme required to release one µg of tyrosine/ml under assay conditions.

Optimization of culture conditions for maximum production of extracellular protease
Optimization of carbon source for extracellular protease production
Various carbon sources such as lactose, sucrose, dextrose, xylose, and galactose were used in the production media at a concentration of 2 % w/v, to check the effect of carbon source on protease production. The supernatant was assayed for protease production.

Optimization of carbon concentration for extracellular protease production
To optimize the concentration of selected carbon source for maximum enzyme production, different concentration of galactose ranging from 1 %, 1.5 %, 2 %, 2.5 %, and 3 % was used in the production media and protease activity was assayed.

Optimization of nitrogen source for extracellular protease production
Various nitrogen sources such as meat extract, yeast extract, malt extract, ammonium chloride, and tryptone were used in the production media at a concentration of 1 % w/v, and the medium was incubated at optimized culture conditions and protease activity was assayed.

Optimization of nitrogen concentration for extracellular protease production
To optimize the concentration of selected nitrogen source for maximal production of enzyme, concentration of yeast extract was varied, i.e., 0.5 %, 1 %, 1.5 %. 2 %, 2.5 %, 3 %, and protease activity was assayed.

Optimization of medium pH and incubation temperature for extracellular protease production

For optimization of pH, production medium with different pH values such as 4, 5, 6, 7, 8, 9, 10 and 11 was inoculated with yeast culture under previously optimized production conditions and the protease activity was determined. To study the effect of temperature, the production medium was incubated at different temperature such as 20 °C, 25 °C, 30 °C, 35 °C and 40 °C under optimized condition and enzyme activity was determined.

Optimization of inoculum age and size for extracellular protease production

The optimum inoculum age for the production of extracellular protease enzyme was determined by inoculating the selected yeast culture in the seed medium. The inoculated seed medium was incubated at 30 °C and then seed aging from 12 h to 32 h was added to the production medium and in order to check the effect of inoculum size on protease production, production medium was inoculated with 1 ml, 1.5 ml, 2 ml, 2.5 ml, and 3 ml, (v/v) of 24 h old seed culture. The production medium was incubated under shaking conditions at 150 rpm at 30 °C for 24 h. The protease activity was then determined using the standard method.

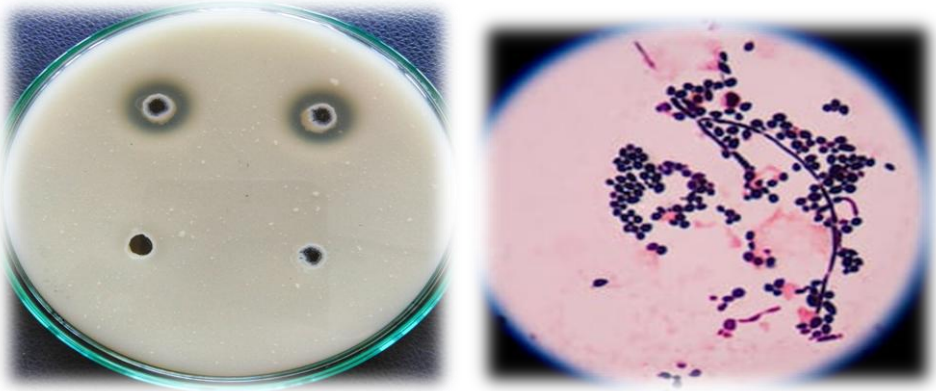
Growth curve and enzyme profile

The growth curve for the yeast isolate was prepared by inoculating the yeast culture into the 20 h seed medium (yeast extract 1 % w/v, peptone 2 % w/v and galactose 2 % w/v). 1.5 ml of seed medium was then inoculated in the production medium and incubated at 30 °C. 1 ml of the inoculated medium was taken in cuvette under aseptic conditions for determining OD (optical density) of the cells at 600 nm at 0 h and then remaining medium was incubated at 30 °C and 150 rpm in incubator shaker. After a regular interval of time i.e. 4 h, cells were drawn from the medium for OD of cells and for determining the enzyme activity of cells up to 32 h. The lag, log, stationary and death phase of the yeast isolate was determined by observing the concentration of cell and enzyme activity of protease from 0 to 32 h was also determined.

RESULTS

Isolation and screening of protease producing yeast

Ten yeasts were isolated from dairy products, collected from Shimla, Himachal Pradesh. These isolates were further screened for extracellular protease production. Out of 10 isolates, only two isolates showed the protease activity. Highest activity of the enzyme was found to be in isolate Y-46 i.e. 0.033 U/ml (Fig. 1a) and this yeast isolate was further selected for the study. The colonies of yeast isolate on YM agar plates were of large size, cream colored and having smooth surface and gave purple colonies on Gram staining (Fig. 1b). The screened isolate was found to be negative for urease activity and positive for catalase activity.



(a) **(b)**

Fig. 1: (a) Yeast isolate Y-46 grown on skimmed milk agar (b) microscopic view (10x*100x magnification)

Optimization of carbon source for extracellular protease production

Carbon source an important component of the medium for sustaining growth and production of enzymes. Lactose, sucrose, dextrose, xylose and galactose at 2 % concentration in growth medium were used and their effect on extracted protease production was studied. The highest protease activity (0.131 U/ml) was observed with galactose and lowest with lactose (Fig. 2).

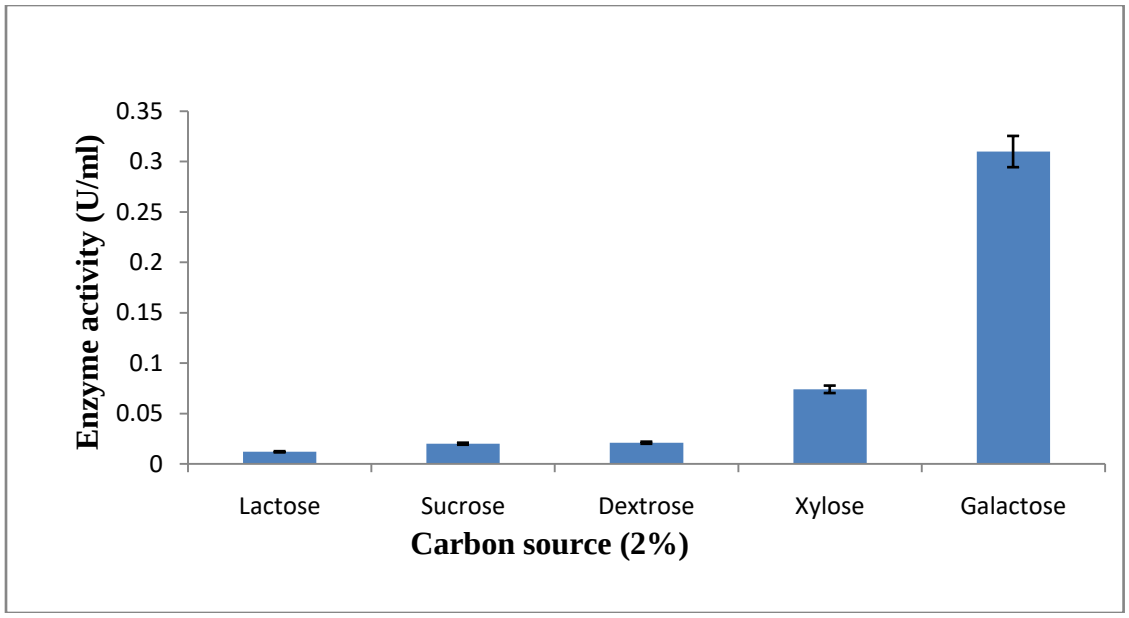


Fig. 2: Effect of different carbon sources on the production of extracellular protease by Y-46 yeast isolate

Optimization of carbon concentration for extracellular protease production

Carbon source optimization revealed the galactose is the best source for the production of protease enzyme by yeast Y-46. Further, the effect of galactose concentration on extracellular protease production was studied and maximum protease activity (0.141 U/ml) was observed in medium containing 2 % of galactose (Fig. 3).

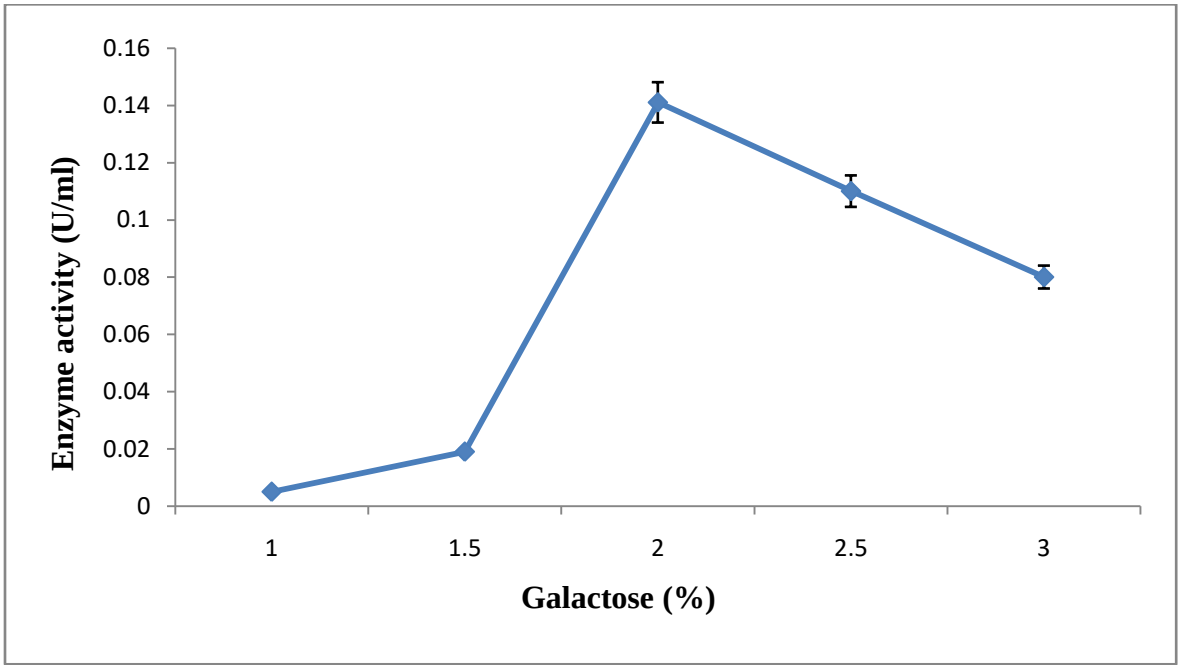


Fig. 3: Effect of different concentration of galactose on the production of extracellular protease by Y-46 yeast isolate

Optimization of nitrogen source for extracellular protease production

The effect of five different nitrogen sources (1 % each) on the protease activity of Y-46 was tested (Fig. 4). The enzyme activity was found to be highest in production media supplemented with yeast extract (0.209 U/ml).

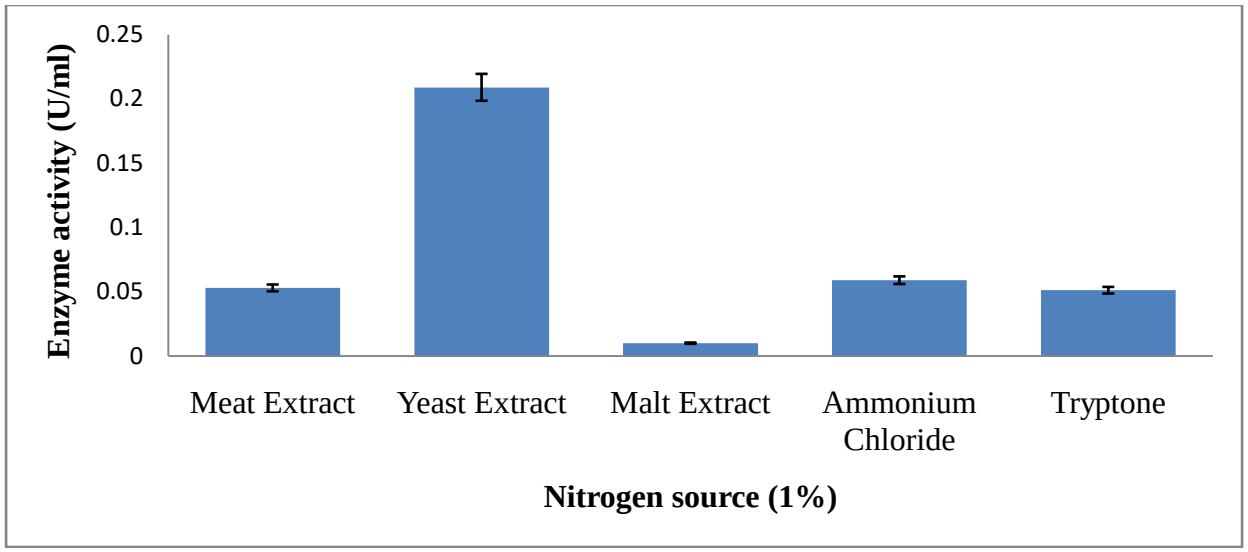


Fig. 4: Effect of different nitrogen sources on the production of extracellular protease by Y-46 yeast isolate

Optimization of nitrogen concentration for extracellular protease production

The concentration of nitrogen source was optimized and maximum activity was found to be 0.243 U/ml in the medium supplemented with 1 % yeast extract (Fig. 5).

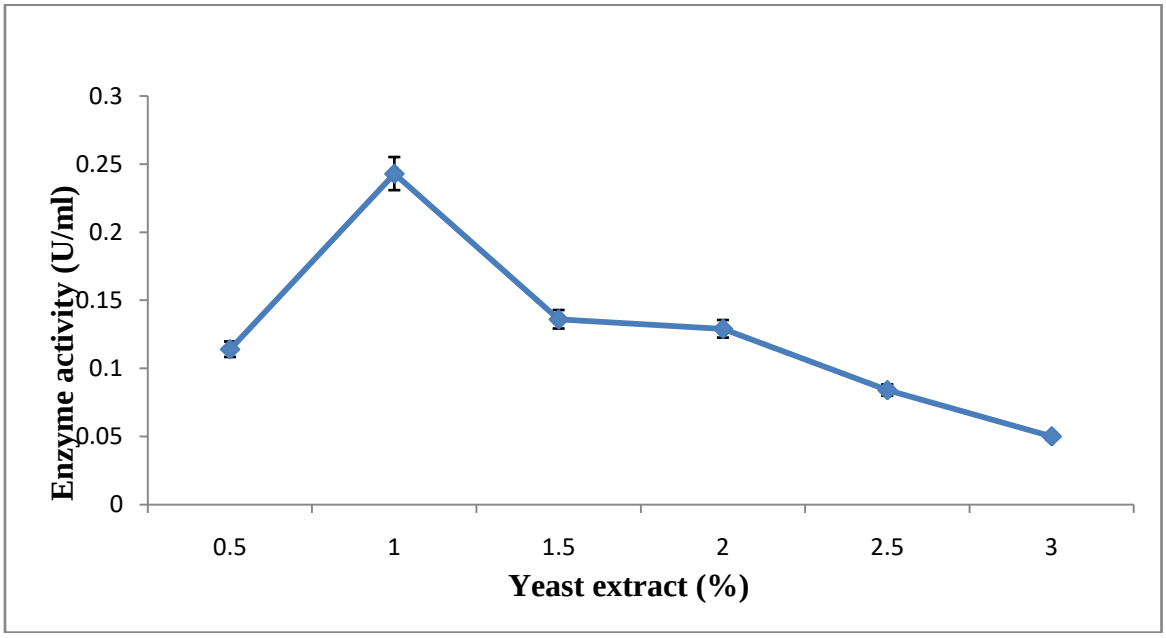


Fig. 5: Effect of varying concentration of yeast extract on the production of extracellular protease by Y-46 yeast isolate

Optimization of medium pH and incubation temperature for extracellular protease production

The production of protease enzyme was studied at different pH and temperature. The production medium having pH of 8 showed the maximum enzyme activity of 0.298 U/ml (Fig. 6a) and this pH was considered as optimized pH for further experiments. To study the effect of incubation temperature on extracellular protease enzyme production, the culture was grown at different temperatures ranging from 20 °C to 40 °C. The production medium incubated at 30°C showed the maximum enzyme production with an enzyme activity of 0.32 U/ml (Fig. 6b).

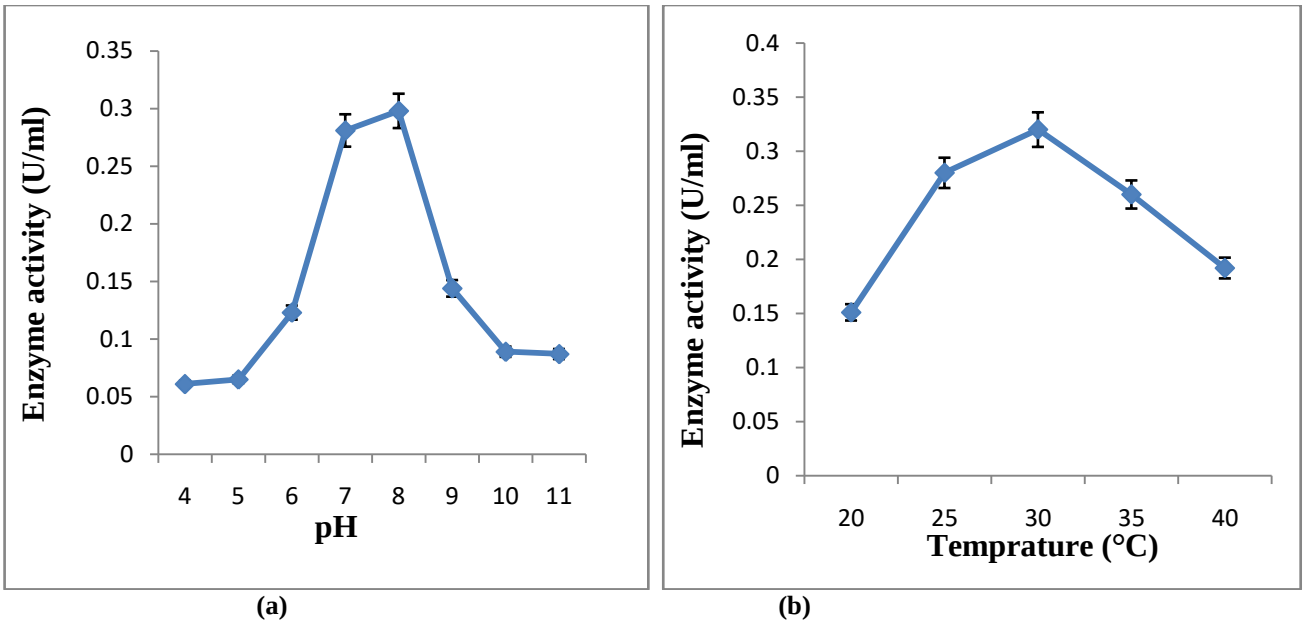


Fig. 6: (a) Effect of pH on the production of extracellular protease (b) Effect of temperature on the production of extracellular protease by Y-46 yeast isolate

Optimization of inoculum age and size for extracellular protease production

The effect of age of inoculum of yeast on extracellular protease production was studied by inoculating the production broth with inoculum culture of varying age and estimating the protease activity from 12 h to 32 h of production. The highest activity of the enzyme was observed in the inoculum age of 24 h with the enzyme activity of 0.372 U/ml (Fig. 7a). Further, the inoculum size was varied from 1 ml to 3 ml in the production medium and the highest activity of protease enzyme was observed in the inoculum size of 1.5 ml with an enzyme activity of 0.393 U/ml (Fig. 7b).

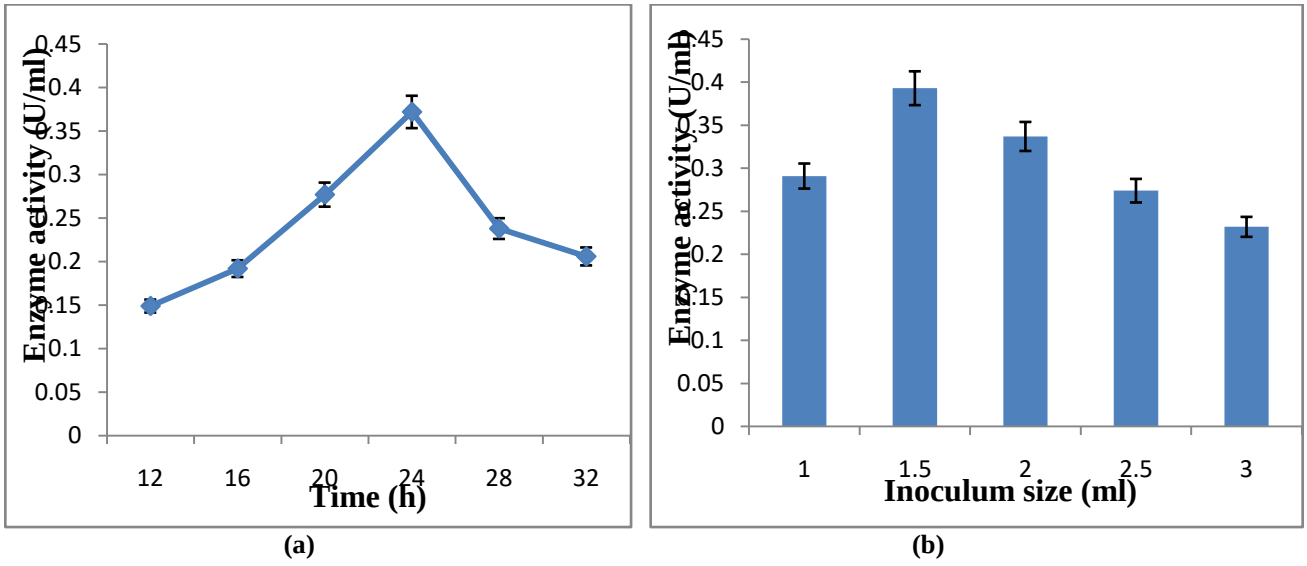


Fig. 7: (a) Effect of inoculum age on the production of extracellular protease (b) Effect of inoculum size on the production of extracellular protease by Y-46 yeast isolate

Growth curve and enzyme profile

The growth profile of Y-46 and extracellular protease production was studied over a period of 32 h. During the lag phase of the growth i.e., from 0-12 h, there was no net increase in cell number as well enzyme activity, when it enters the log phase or exponential phase of growth i.e., from 12-24 h, cell begin multiply and showed the highest activity of the protease enzyme i.e., 0.42 U/ml at 24 h. After a certain period, the rate of division equal to the rate of cell death which results in

stationary phase. The stationary phase of Y-46 yeast isolate lies between 24-28 h and the extracellular protease enzyme activity at this phase was 0.36 U/ml. The final stage was the death phase i.e., from 28-32 h with further decrease in activity as shown in Fig. 8. After optimization of culture conditions for extracellular protease enzyme by Y-46 yeast isolate, enzyme activity was increase by 12.73 fold.

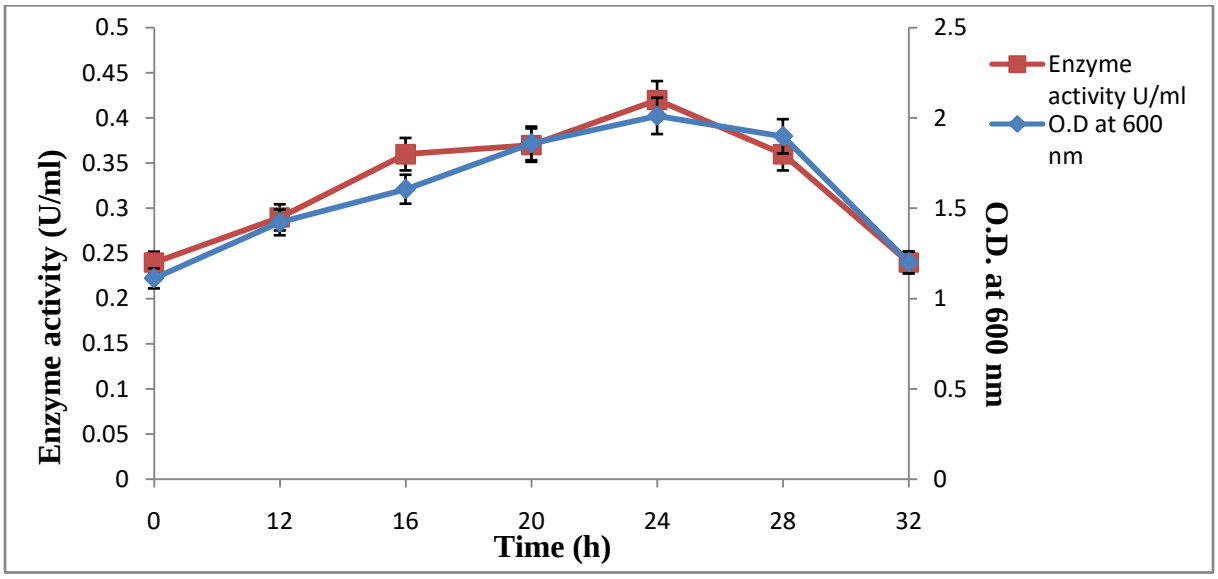


Fig. 8: Growth curve and enzyme production profile of Y-46 yeast isolate

DISCUSSION

Proteases represented one of the three largest groups of industrial enzyme and account for 60 % of the total worldwide sale of enzyme. They are ubiquitous in all living beings and show a vast diversity of physiochemical and catalytic properties. In order to increase the production of protease enzyme, different optimization experiments were carried out using the classical method by varying one parameter at a time and keeping the others constant at pre-determined levels.

In the present study, five production media were screened for highest protease activity. Out of five production media, M-4 (galactose 2 %, peptone 2 % and yeast extract 1%) showed highest protease production i.e.0.033 U/ml and with the increase in amount of nitrogen and carbon source the enzyme activity decreased. The optimum media pH for protease production Y-46 was found to be 8. At a higher pH metabolic action of the yeast could have been suppressed thus decreasing the enzyme production. Similar trend have been observed in protease by *Bacillus* sp. [10, 11] and a metaloprotease secreted by *Salinivibrio* sp. strain AF- 2004 [12]. Y-46 also showed a sharp increase in protease production as the incubation temperature was increased from 25 °C to 30 °C. Beyond 30 °C there was a steady decline in protease production with the enzyme activity remaining fairly stable till 35 °C. Similar temperature ranges for the production of protease have been reported in *B. pumillus* ATCC7061 [13] and also in *B. cereus* [14].

Among the different carbon sources, 2 % galactose was found to be the most preferred carbon source for protease production by Y-46. Similar preference towards galactose was reported by Pastor et al. [15] and Sen and Satyanarayana [16]. The protease yield decreased with increase in concentration of galactose in the production medium. This observation is consistent with the effect of galactose on protease production by previously reported strain [17, 18].

Organic nitrogen source (yeast extract) was found to be better source for protease production in Y-46 than the other nitrogen sources. A similar trend was reported in case of *B. pumilus* protease [19] and serratiopeptidase enzyme of *S. marcescens* [20] and protease from *Serratia marcescens* PPB-26 [21]. More specifically 1 % yeast extract showed the best results and protease activity started decreasing gradually with increase in the yeast extract concentration till 2.5 % beyond which the sharp decline in the activity was recorded. The Y-46 exhibited maximum protease activity when the production medium was seeded with 1.5 ml inoculum size. Use of inoculum more than 1.5 ml resulted into a decrease in the enzyme activity which might be due to faster depletion of nutrients in the medium. Y-46 showed maximum protease activity after 24 h of incubation. A similar trend was observed with protease from *S. marcescens* PPB-26 [21].

CONCLUSION

This study sought to optimize various physicochemical parameters for the optimum extracellular protease production by yeast isolate Y-46. The result of the present work showed 12.73 fold increases in protease activity and suggested that the components of production media, inoculum size, inoculum age, pH, and incubation temperature, are responsible for the enhancement of protease production by yeast isolate Y-46. The protease from yeast isolated from dairy products can be safely used in various applications including foods, bakeries and breweries and could also be explored for other industrial applications.

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