

Original Article

SCREENING, MEDIA OPTIMIZATION AND PARTIAL PURIFICATION OF PROTEASE BY TRICHOSPORON JAPONICUM VITVK1

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ABSTRACT

Objective: The main aim of the study was to screen, optimize and partial purification of protease from fungi. The isolated fungi were screened for protease activity on a modified SDA plate and subjected to media optimization. Comparative study of free and immobilized cells was done.

Methods: A partial sequencing of the fungi was done. The process parameters were examined by the classical method and Plackett Burman method, including carbon, nitrogen sources, pH, temperature, agitation and inoculum size were optimized. The enzyme was subjected to step by step purification process by ammonium sulfate precipitation.

Results: The total protein concentration was determined and the specific activity was also performed by casein hydrolysis assay. The Plackett-Burman method shows fructose, peptone pH are significant factors for optimized protease production. Immobilized cell shows reduction in protease production. In optimum conditions the protease produced was 4758.4U/mg. Protease was partially purified at 60% ammonium sulfate saturation.

Conclusion: *Trichosporon japonicum* VITVK1 estimated as a strong candidate for the production of Protease.

Keywords: Protease, Fungi, *Trichosporon japonicum*, Screening, Optimization.

INTRODUCTION

Protease accounts for nearly 60% of the industrial enzyme market and has wide applications in many industries viz., textiles, detergents, food processing, especially for cheese ripening, meat tenderizing, animal nutrition, pharmaceuticals, paper industry and the food industry. These enzymes are also reported having a significant role in the development and manifestation of dreadful diseases such as AIDS and cancer [1].

The ability to secrete glycosylated proteins makes filamentous fungi attractive hosts for recombinant protein production. They have been widely used for the production of heterologous proteins as well as homologous proteins. Production levels of grams per liter have been reported. However, compared with homologous fungal protein productions, the production levels of heterologous non-fungal proteins usually do not exceed a few tens of milligrams per liter. In attempts to improve the yield of heterologous proteins in fungal fermentations, several strategies have been developed, such as using gene fusion, protease deficient mutants, strong fungal promoters and efficient secretion signals and the introduction of a large number of gene copies.

In nature, filamentous fungi are able to utilize a great variety of carbon and nitrogen sources by secreting a wide range of different enzymes in large amounts into their environment. Unfortunately, the degradation of heterologous proteins by extracellular proteases secreted by fungi into culture media has been considered a major problem in the efficient production of recombinant [2]. There is very less knowledge of productivity of protease from *Trichosporon* species. Hence the main aim of the study was to screen, optimize and partial purification of protease from *Trichosporon japonicum* VITVK1.

MATERIALS AND METHODS

Screening for protease production

Test fungi VITVK1 was tested for their ability to produce alkaline protease using a solid agar plate assay on SDA. The fungi were grown on SDA containing 1% casein as protein substrate and the plates were incubated at 28°C to allow the growth of the test fungi

for 2 days and was examined for the formation of the zone of clearance around the colony [3].

Sequencing of fungi

Preparation of template DNA

Pure fungal culture was used for identification. Colonies were picked up with a sterilized toothpick, and suspended in 0.5 ml of sterilized saline in a 1.5 ml centrifuge tubes and centrifuged at 10,000 RPM for 10 min. After removal of supernatant, the pellet is suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA). Incubated 56°C for 30 min and then heated 100°C for 10 min. After heating, the supernatant can be used for PCR. Total genomic DNA was isolated using the phenol chloroform method. PCR amplification of 18S rDNA was carried out using 518F universal forward primer CCAgCagCCgCggTAATACg and 800R universal reverse primer TACCaggTATCTAATCC for sequencing reference and 27F universal forward primer AgAgTTTgATCMTGGCTCag and universal reverse primer 1492R TACggYTACCTTgTTACgACTT for PCR amplification. The PCR product was detected by agarose gel electrophoresis. Sequencing was performed using big dye terminator cycle sequencing kit (Applied BioSystems, USA) [5].

Optimization of media

Classical method

In this method, the variables are changed one at a time, all the other parameters are kept constant. The effect of each parameter is then individually analyzed. For production of protease by fungi, the main parameters are determined to be the carbon source, nitrogen source (organic and inorganic), pH, temperature, inoculum size and agitation (RPM).

The carbon sources investigated were ranged from 1 to 2% for glucose, fructose, starch, dextrose, and maltose. The solutions were monitored for protease activity daily over a period of five days. The component with the highest result was chosen to be the best. Inorganic nitrogen sources investigated included ammonium chloride, ammonium nitrate, ammonium sulfate, and potassium chloride. Organic nitrogen sources studied were malt extract, Peptone, tryptone and yeast extract. The concentrations used for both organic and inorganic nitrogen sources were 1% and 1.5%.

The temperatures used for incubation were 0°C, 20°C, ambient temperature (around 28-30°C), 40°C and 55°C. The pH ranges used were 3 to 10. Optimum RPM was also investigated with 0 to 150.

Plackett-burman method

The Plackett-Burman method allows for the screening of the effect of k variables. The variables have a high (denoted by +1) or a low (denoted by -1) value. This method enables us to determine the most significant factors. The variables used in this study were concentration of the carbon source, concentration of organic nitrogen, concentration of inorganic nitrogen, temperature, and pH.

Immobilization of fungi by sodium alginate

3 days old fungal culture was centrifuged at 10000 RPM for 10 min at 4 °C and resuspended the pellet in 0.9% saline then sodium alginate was added. With the help of a syringe slowly drop the sodium alginate into a beaker containing calcium chloride (0.3M). Incubated over-night in the calcium chloride solution. Drained the solution and washed with distilled water [6].

Extraction of protease

By ammonium sulphate precipitation

The 60% and 80% (w/v) solid ammonium sulfate was added to the protein solution at 4°C while stirring. After the salt has dissolved, continue stirring for 30 min was allowed and equilibrium was reached between dissolved and aggregated proteins and centrifuged at 12,000 RPM for 10 min at 4°C. At higher salt concentrations somewhat more, centrifugation may be needed.

RESULTS

Sequencing of fungi

Genomic DNA was isolated and subjected to PCR analysis using universal primers and the DNA obtained was found to have 1800bp. The aligned sequence of the amplified 1800bp 18S rDNA fragments from the isolate species *Trichosporon* was submitted to Genbank. The sequence of the isolate was found similar with the species *Trichosporon japonicum* AUMC 7800. Therefore the fungi were assigned and confirmed as *Trichosporon japonicum* VITVK1. The sequence has been submitted to GenBank accession number KM464554.

Classical method

Incubation period

The highest protease activity was found at 2nd day was 45U/L. The experiment was performed in two sets A and B. Bradford assay was also performed for total protein estimation.

Carbon sources

The highest protease activity was found at 2nd day on 2% fructose carbon source. As we can see from the protease activity graphs fructose give a significantly higher activity at most concentrations. At 2% fructose gave the highest activity with activity of 904U/L and 680U/L. And protein concentration was 0.148mg/mL and 0.181mg/mL respectively.

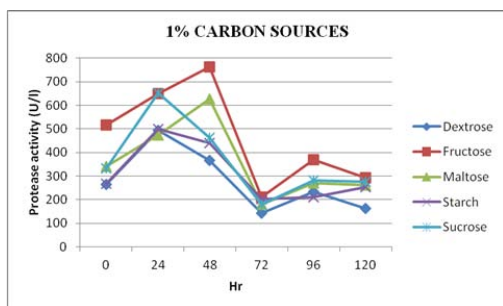


Fig. 1: Graph for carbon sources optimization shows protease concentration in U/L

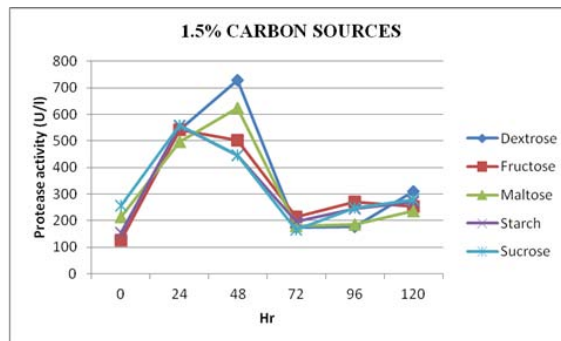


Fig. 2: Graph for carbon sources optimization shows protease concentration in U/L

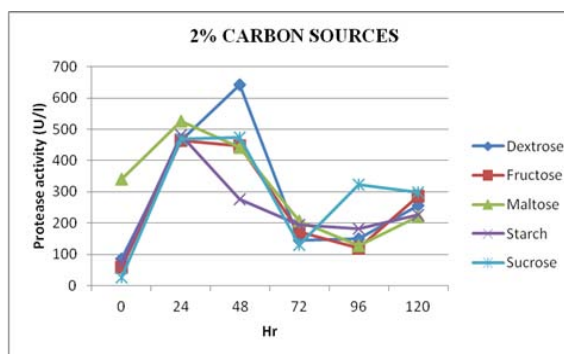


Fig. 3: Graph for carbon sources optimization shows protease concentration in U/L

Nitrogen sources

Organic nitrogen

The highest protease activity was found at 4th day on 1.5% peptone 4653 U/L from set A. Peptone gives a high activity in all cases and 2926U/L from set B. Tryptone is the next best source for organic nitrogen with values that are close to that of peptone.

The experiment was performed in two sets A and B. Bradford assay was also performed for total protein estimation. Protein was 0.28mg/mL and 0.30mg/mL, respectively, for peptone for set A and set B.

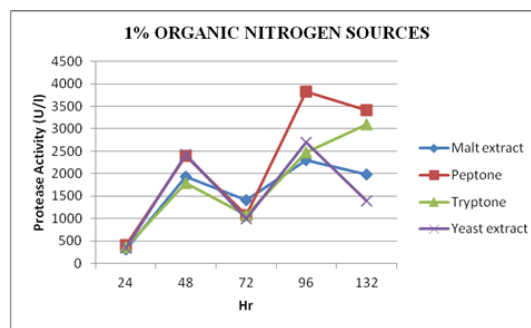


Fig. 4: Graph for organic nitrogen sources optimization shows protease concentration in U/L

Inorganic nitrogen sources

The highest protease activity was found at 3rd day on 1.5% ammonium sulfate was 3296 U/L and 2981 U/L from set A and set B respectively. The experiment was performed in two sets A and B. Bradford assay was also performed for total protein estimation.

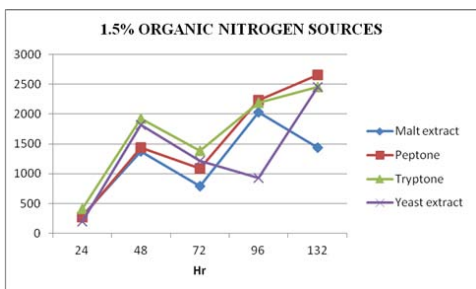


Fig. 5: Graph for organic nitrogen sources optimization shows protease concentration in U/L

In two cases out of four ammonium sulphates give the highest values of protease activity from set A & set B is 3297U/L and 2982U/L, respectively. Total protein concentration for set A and set B was 0.03mg/mL and 0.07mg/mL respectively.

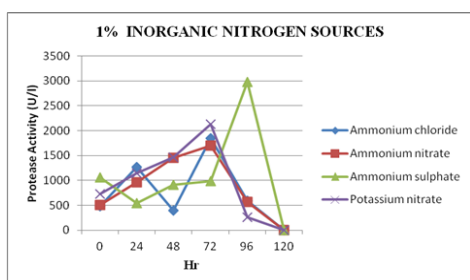


Fig. 6: Graph for inorganic nitrogen sources optimization shows protease concentration in U/L

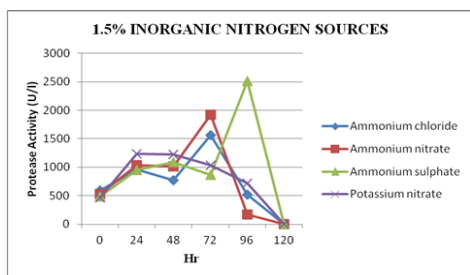


Fig. 7: Graph for inorganic nitrogen sources optimization shows protease concentration in U/L

pH

The highest protease activity was found at the first day at pH 7. The experiment was performed in two sets A (849U/L) and B (687U/L). The highest activity seen at pH 3 for set A is not seen in set B and so is not considered. Bradford assay was also performed for total protein estimation. Total protein concentration for set A and set B was 0.04mg/mL and 0.15mg/mL, respectively, for pH 7 at the first day.

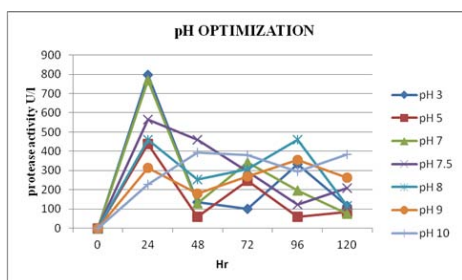


Fig. 8: Graph for pH optimization shows protease concentration in U/L

Temperature

The highest protease activity was found at 2nd day at 40°C. The experiment was performed in two sets A and B. In set A the highest activity recorded, was at 40°C at 1785U/L and was closely followed by the control at around 1400U/L. In set B the control showed an activity of 1809U/L followed by the sample at 40°C showing 1693U/mL. This shows that the sample at 40°C is optimum temperature for the production of protease. Bradford assay was also performed for total protein estimation.

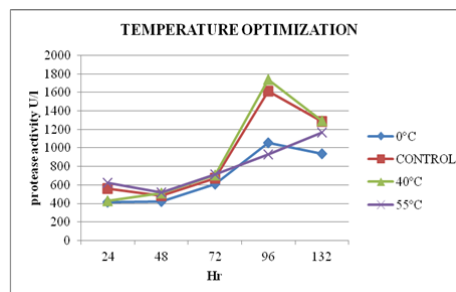


Fig. 9: Graph for temperature optimization shows protease concentration in U/L

RPM

The highest protease activity was found at the first day on 125rpm. The experiment was performed in two sets A and B. In set A the highest activity recorded was at 125rpm at 785U/L. In set B at 125rpm showed an activity of 630U/L. This shows that the 125rpm are both optimum temperatures for the production of protease. Bradford assay was also performed for total protein estimation. Total protein concentration for Set A and Set B were 0.56mg/mL and 0.55mg/mL.

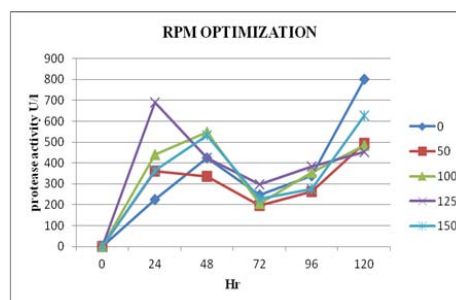


Fig. 10: Graph for RPM optimization shows protease concentration in U/L

Inoculum size

The highest protease activity was found at 2nd day on 4% (v/v) inoculum size at from set A & set B are 808U/L and 847U/L. The experiment was performed in two sets A and B. Bradford assay was also performed for total protein estimation. Total protein concentration was for set A and set B were 0.24mg/mL and 0.17mg/mL, respectively.

Plackett-burman method

On Plackett-Burman we get three optimum parameters A-Fructose, B-Peptone, G-RPM other parameters which show the positive effect are pH, inoculum size. Parameters shows negative effects are ammonium sulfate and incubation period.

The Model F-value of 15.91 implies the model is significant. There is only a 0.21% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicates model terms are significant

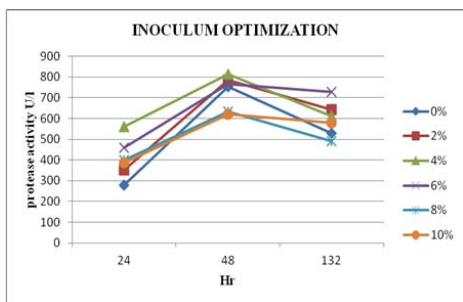


Fig. 11: Graph for inoculum size optimization shows protease concentration in U/L

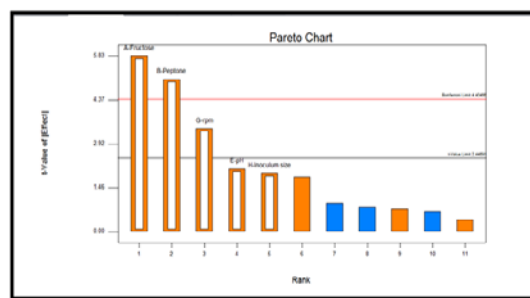


Fig. 12: Pareto chart for media optimization (Plackett- Berman method) A, B, G are significant model terms.

Table 1: Anova table shows that fructose, peptone and pH are significant for protease production

Analysis of variance table [Partial sum of squares - Type III]					
Source	Sum of Mean Fp-value Squares	df	Square	Value	Prob > F
Model	1.96	5	0.39	15.91	0.0021
A-Fructose	0.84	1	0.84	34.02	0.0011
B-Peptide	0.63	1	0.63	25.72	0.0023
E-pH	0.11	1	0.11	4.36	0.0819
G-RPM	0.29	1	0.29	11.74	0.0140
H-Inoculum size	0.092	1	0.092	3.72	0.1021
Residual	0.15	6	0.025		
Cor Total	2.11	11			

Comparison between immobilized cell and free cell

The protease activity and Bradford assay were performed at 24 hrs, 48 hrs and 132 hrs respectively. The highest activity was found at 48 hr was 638U/L in set A from free cells. Total protein concentration 0.28mg/mL and 0.30mg/mL for set A and set B respectively.

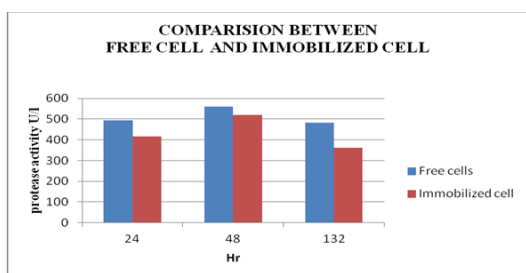


Fig. 13: Graph for comparison of immobilized and mobilized cells shows protease concentration in U/L

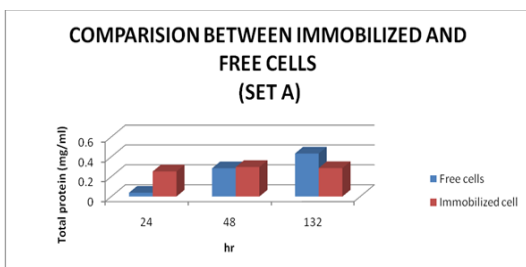


Fig. 14: Graph for comparison between immobilized and free cells shows total protein concentration in mg/mL

Purification

Ammonium sulphate purification

Protease was partially purified at 60% and 80% (w/v). Protease concentration and total protein were estimated by the Bradford assay.

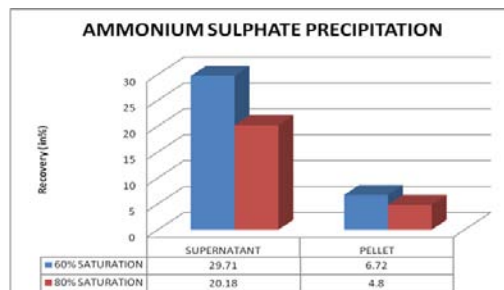


Fig. 15: Recovery by ammonium sulfate precipitation at 60% and 80% saturation

DISCUSSION

We have screened fungi VITVK1 and identified as *Trichosporon japonicum* VITVK1 for protease production. The isolate belongs to basidiomycetes there are various fungi which produces extracellular protease. There is less literature available for protease producing *Trichosporon* species. As far author knows there is no literature available for optimization of *Trichosporon japonicum* species for protease production.

The addition of simple carbon, nitrogen source in the basal media shows increase in protease production and maximum protease production was 904U/L at 2% fructose and an average value was 761.5U/L at 1% fructose, 3825.17U/L at 1.5% peptone as organic nitrogen source and 2981.68U/L 1% ammonium sulfate as an inorganic nitrogen source. Protease production was observed for RPM and results is 689.7U/L at 125 RPM, 24 hours and 799.3U/L at 0 RPM, 120 hours which show positive effects of RPM. Optimum temperature was reported as 40°C was an average activity was 1738.5U/L similarly for 4% inoculums size activity was 814.5U/L. A discrepancy of pH was due to incorrect pH measurements yet we rely on over observation with control (pH 7 was 767.98U/L) not at pH 3 which was 795.6U/L because at pH 7.5 shows maxima and other lower pH values are not shown that trends [Fig. 8].

Our Plackett Burman analysis reveals that fructose, protease, RPM, pH and inoculums size are the factors needed for optimized

production of protease. The comparison between immobilized and mobilized cell shows that immobilization causes a negative effect on protease production. We optimized carbon sources, nitrogen sources, temperature, pH, inoculum size and RPM. The optimized protease production was 4758.4U/mg. Whereas the protease production from *Aureobasidium pullulans* was 632.1U/mg [7] and 70 U/l from *geotrichum capitulum* [8].

CONCLUSION

We optimized the various components of the media and also compared the immobilized and free cell. Classical and Plackett-Burman method was used for optimization of the media. The parameters significant during optimization are carbon sources, organic nitrogen sources, agitation. The protease unit produced per liter was reliable. The protease was partially purified precipitation method by the ammonium sulfate with 60% saturation giving highest protease activity.

CONFLICT OF INTERESTS

Declared None.

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