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Original Research Article

DESIGN OF CULTURAL CONDITIONS FOR ENHANCEMENT OF ALKALINE PROTEASE PRODUCTION

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ABSTRACT

Among the various industrial enzymes studied, proteases are the most widely explored enzyme. They occupy the top position in terms of the amount of enzyme produced commercially. In the present study, soil samples collected from various locations around Manipal, Udupi District, Karnataka were screened for proteolytic activity. Casein agar medium was used as basal medium. The isolate showing highest activity during primary screening was further studied for proteolytic activity. Among the isolates screened, KWF-2 obtained from drains of kitchen waste, showed significant proteolytic activity. Therefore, it was further studied by shake flask culture method. Protease yield was improved through cultural condition optimization. These conditions were optimized by one-factor-at-a-time method. The protease was found to be an alkaline protease. Due to the promising result, further studies on characterization of the enzyme are being carried out.

Key words: Screening, Protease, Optimization.

INTRODUCTION

Among the various ecosystem on earth, soil is considered to be the richest source of native microbes observed in terrestrial environment. A number of commercially useful enzymes including proteases have been traditionally produced using microbes isolated from soil. Each environment has its own unique characteristics and hence there is a wide difference in microbial diversity observed at various localities [1]. Microbes distributed in the soil, produces a number of extracellular enzymes which mainly helps microorganism to breakdown complex organic matter into their simplest form. This helps microbes to meet their nutritional requirements [2]. Rapid change in the climate along with urbanization and change in agriculture practices affects microbial population observed at a particular location. Therefore, microbial diversity changes over a time with respect to the changing flora and fauna. Even in case of under reported records it is observed that there are at least 10^8-10^{10} microbes present in 1 g of soil. Therefore there is always a possibility that the number of microorganisms found in a given sample of soil will far exceed the expected limit. Microbes found in natural habitat constantly try to evolve so that they are able to produce extracellular enzymes spending the least possible energy. Hence, through traditional screening method and natural selection it is possible to isolate potential microorganisms that could be useful at industrial level [3,4]. A number of microbial products including microbial derived enzymes find various industrial applications. Among the industrial enzymes, proteases are the most widely explored. These are useful in detergent and textile industries. Of late, proteases are increasingly studied for therapeutic application[5].

In the present study, cultural conditions for protease production by an isolate KWF-2 obtained from one of the soil screening studies was carried out. Through one factor at a time method of optimization studies the enzyme yield was improved significantly.

MATERIALS AND METHODS

Chemicals and Reagents

All the dehydrated microbiological media were procured from Himedia. Ammonium sulphate, Magnesium sulphate, Calcium carbonate, Folin- Ciocalteu's phenol reagent and other Fine Chemicals procured from Merck Pvt. Ltd. Mumbai, India.

MICROORGANISM AND CULTURAL CONDITIONS

Sample collection, primary and secondary screening

Soil samples from various locations around Manipal, Udupi district, India were collected in sterile tubes. Samples from

twelve different locations were screened for proteolytic activity using casein agar medium. Samples were taken from a depth of 5-10 cm. Pour plate technique was followed. Stock was prepared by suspending one gram of soil in 0.85% saline. 1 mL from the stock was transferred to 9 mL of sterile saline to get first dilution. Similarly six dilutions were made. One mL from fifth and sixth dilutions were aseptically added to molten casein (1%) agar (15 mL) and poured into sterile petri plates. The plates were incubated at 27°C for three days. Fungal colonies that appeared on the plates were streaked once again on casein agar (1%) and incubated for 48 h. The isolates showing clear zones were considered positive. Among them, KWF-2 showed maximum clear zone. Therefore, KWF-2 was selected for further studies. The selected isolate was sub-cultured on Sabouraud dextrose agar slants and incubated at 27°C for ten days. After complete sporulation, the isolates were preserved at 4°C in refrigerator and were used during further studies whenever needed.

Optimization of cultural conditions

Protease yield of KWF-2 was improved through cultural condition optimization by one-factor-at -a-time design (OFAT). Glucose-Yeast extract medium was used as inoculation medium. Growth from a 10 day old slant was transferred to inoculation medium (50 mL) consisting of glucose (2 %w/v), yeast extract (1% w/v), K_2HPO_4 (0.1% w/v), KH_2PO_4 (0.1% w/v) and $MgSO_4$ (0.02 % w/v) with pH adjusted to 7 and incubated on a rotary shaking incubator at 150 rpm at 28°C for 48 h. Production medium remained same except for the presence of casein at 1.5% w/v was seeded with 4 h old inoculum at 10% level. The inoculated flasks were kept on a rotary shaking incubator (150 rpm) at 28°C for 7 days for production of protease. The effect of initial pH *viz.*, 3, 5, 7 and 9 of the production medium on enzyme yield was studied by adjusting the production medium to the required pH using1N NaOH and 1N HCl and inoculating them for protease production. Samples were withdrawn every 24 h for six days and the amount of tyrosine liberated was estimated. After identifying optimum pH, the effect of various carbon sources *viz.*, dextrose, sucrose, fructose and soluble starch at 1% level was studied. Effect of various concentrations of $MgSO_4$ and $CaCO_3$ was studied by including each of these metals at 0.01, 0.05 and 0.1 %w/v (Table 1).

Table.1.Various combinations of Mg^{2+} and Ca^{2+} metal ions

Trace elements	Combination			
	1	2	3	4
$MgSO_4$	0.01%	0.1%	0.05%	0.1%
$CaCO_3$	0.1%	0.01%	0.05%	0.1%

Protease activity was measured by Folin-Ciocalteu's method with tyrosine being used as standard.

Assay of protease

Enzyme activity was measured by tyrosine method. The enzyme solution (1 ml) was incubated with casein (1% w/v). Amount of tyrosine liberated was estimated by adding Folin-Ciocalteau reagent and the absorbance measured at 660 nm. One unit of protease activity (U) is defined as the amount of enzyme which releases one µg of tyrosine per min at 37° C [5].

RESULTS FOR BIOLOGICAL ACTIVITIES

Primary screening

Proteolytic activity of KWF2 was far higher than the other isolates (Table 2).

Table.2.Protease activity of the isolates

Isolate	Protease activity (zone of activity in cm)
FL2b	0.5
FL3a	1.4
FM1a	0.3
FM2	0.3
KWF-2	2.9
MC1	2.7
MC2	2.4
MNSS1	1.0

Among the isolates obtained during screening, KWF-2 showed the highest activity with zone of activity being 2.9 cm (Fig. 1).

Fig.1.Proteolytic activity of KWF-2

The isolate KWF-2 had maximum proteolytic activity with a clear zone of 2.9 cm, where as isolates FM1a and FM2 showed the least activity with zone of activity being 0.3 cm. Since prominent proteolytic activity was observed for KWF-2 among the isolates tested, they were selected for optimization studies.

Tyrosine standard curve

A series of tyrosine concentration was prepared and its absorbance was measured (Fig. 2).

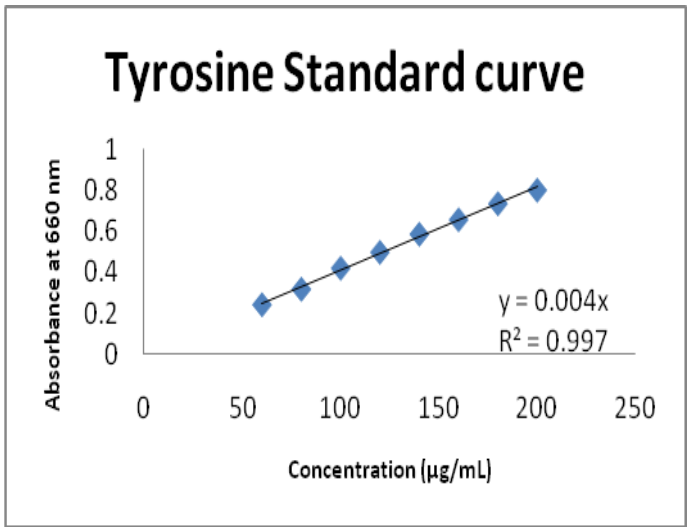

Fig.2.Tyrosine standard curve

(A serial concentration of tyrosine was prepared from the stock concentration of 1 mg/mL.Amount of tyrosine was estimated colorimetrically at 660 nm by Folin-Ciocalteu's method.) Protease activity of the culture supernatant was directly read from the standard curve.

Optimization of growth conditions

The type and yield of protease is known to be highly dependent on pH of the production medium. Therefore, the effect of initial pH of the production medium on protease production by KWF-2 was evaluated. The study showed that enzyme yield was better in alkaline conditions. There was appreciable fall in enzyme yield under acidic conditions especially below pH 5.0. Among the various pH tested, maximum yield was observed at pH 9.0. At pH 9.0, amount of tyrosine released was 52.4mg, while with pH 3 it was observed to be 22.7 mg (Fig. 3). Under alkaline conditions the protease yield was better.

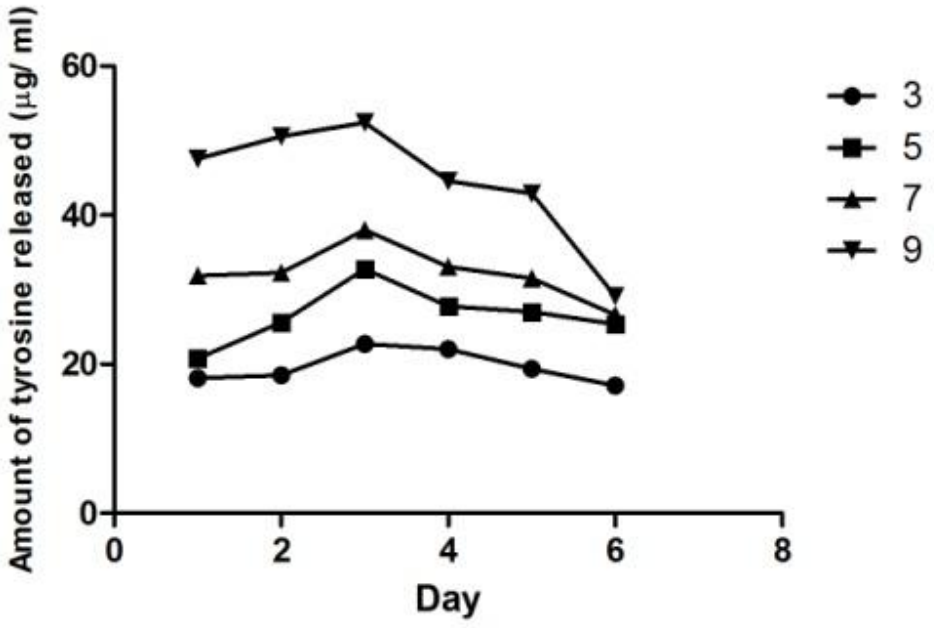

Fig.3.Effect of initial production medium pH on protease yield

The study on effect of nutrient supplementation showed that among the various carbon sources tested on protease production, dextrose at 1% w/v increased enzyme yield compared to other carbon sources (Fig. 4).

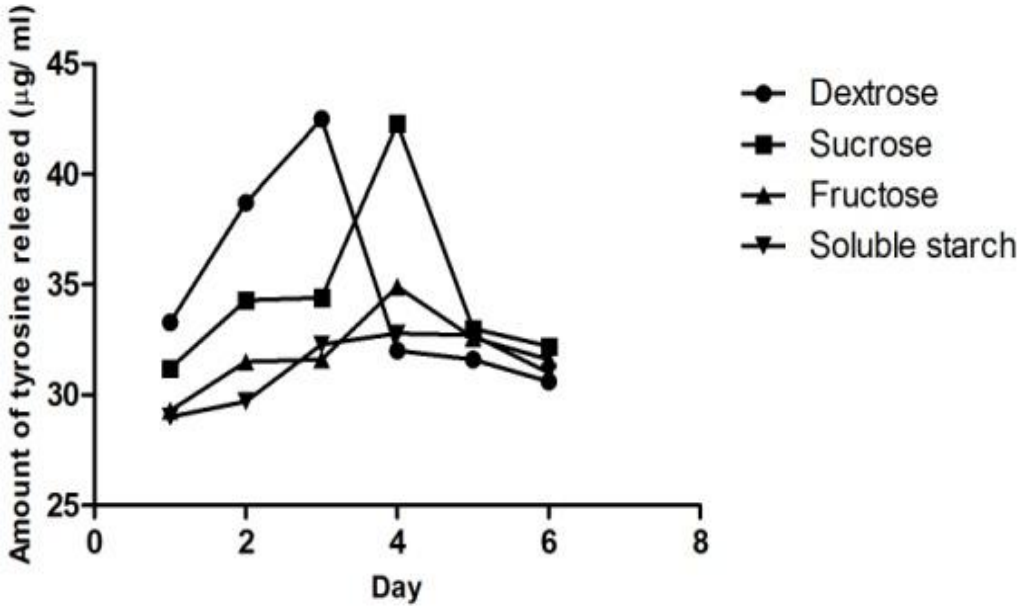

Fig.4.Effect of carbon supplements on protease yield

A combination of Mg^{2+} and Ca^{2+} at concentration of 0.1 and 0.01% w/v respectively improved protease yield (Fig. 5).

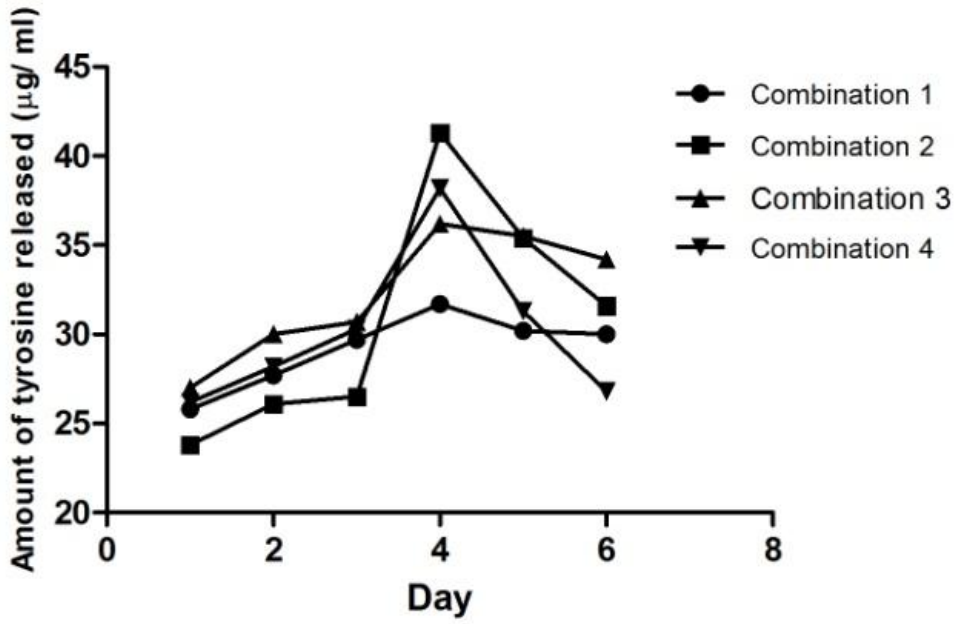

Fig.5.Effect of metal ions

DISCUSSION

Among the various isolates obtained through screening, KWF-2 was found to be a promising isolate. Therefore, the cultural conditions were optimized to improve protease yield.The protease production by this isolate was not under catabolic repression. This can be observed from the increase in enzyme yield when glucose was used as a carbon source. *A. oryzae* and *A. carbonarius* also show a similar dependence on glucose for its enzyme yield [6, 7]. pH is an important factor that affects production of a metabolite besides affecting the microbial growth. In the present study, maximum yield of protease was found in alkaline conditions. *Conidiobolus coronatus* and *Aspergillus tamari* has been reported to produce maximum amount of alkaline protease when grown on a medium with initial pH 9.0 [8, 9]. This shows that protease from KWF-2 is an alkaline protease. Alkaline proteases are useful in tanning, leather and detergent industries. Calcium plays an important role in increasing the protease yield. In organism such as *B. cereus* and *B. licheniformis*, Ca^{2+} is reported to increase the protease yield significantly. In case of *P. aeruginosa,* addition of either calcium or magnesium causes significant increase in protease production. This could be due to the role of calcium in maintaining conformational structure [10-13].

CONCLUSION

Among the various isolates isolated, KWF-2 showed maximum protease activity. Due to high protease activity and its commercial and therapeutic importance, the cultural conditions were optimized to improve protease yield. Currently, efforts are made to characterize the isolated organism and the enzyme.

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