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OPTIMIZATION OF CONDITIONS FOR PRODUCTION OF KERATINASE BY *ASPERGILLUS FLAVUS* BY SUBMERGED FERMNETATION

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ABSTRACT

The fungal strains were isolated from the soil samples collected from different localities in Ernakulam and Thrissur districts. The keratinolytic nature of the fungus made it easy to isolate using Vanbreuseghem's hair baiting technique. The fungi collected were cultured in keratin agar medium. The keratinolytic nature of the fungi were noted by observing the zone of clearance in the keratin agar medium. In this study, out of 350 fungi isolated, 42 presented clear zones in the keratin agar media. The selected fungi were tested for enzyme production by submerged fermentation using keratin substrate. The highest production of extracellular keratinase was shown by the fungus identified as *Aspergillusflavus* Optimization of conditions like incubation period, temperature, pH, supplementary sources etc. for maximum production of enzyme by Aspergillusflavus was done. The maximum production of the enzyme was obtained ,at 6thday of incubation. The maximum enzyme production by the fungus *A. flavus* S125 was observed at 55°C and pH 9. In the study *A. Flavus* cultured on a simple medium supplemented with chicken feather, produced a protease mixture with high keratinolytic activity at high pH, and was very effective in feather degradation, suggesting its potential use in biotechnological processes involving keratin hydrolysis. Moreover, the serine-type keratinase produced by the strain was active over a wide range of pH and temperature.

KEYWORDS: Keratinolytic fungi, Submerged fermentation, *Aspergillusflavus,* Keratinase, Optimization, Hair baiting.

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INTRODUCTION

With the objective of obtaining high yield of keratinase by submerged fermentation, factors influencing the production of the selected fungal strain *Aspergillusflavus*S125 were studied. The factors influencing the production of keratinase were studied one by one examining one factor at a time. The optimized condition with respect to a factor was incorporated in the experiment for the optimization of the next factor. The experiments were done in triplicates.

MATERIALS AND METHODS

Method of preparation of medium
2 gm. of keratin substrate was added to 100ml. of mineral salt solution and the pH was adjusted to 8. The medium was sterilised by autoclaving at 121˚C for 15 min.

Inoculum Preparation
Spore suspension of the fungal isolates was prepared by adding 10 ml. of normal saline to 5 days old fungal isolates growing on SDA agar slants. Final concentration of the spore suspension was adjusted to about $2x10^8$/ml (using a hemocytometer).

Optimization of conditions for enzyme production - Incubation period
The flasks were inoculated with spore suspension at a level $2x10^8$ and were incubated at 37^o C in an incubator shaker (Labline). The culture supernatant was collected at various intervals of incubation and the production of the enzyme was estimated.

Temperature
The effect of temperature on the production of keratinase enzyme was studied using the same medium as in the previous experiment. The medium was inoculated and incubated at different temperature and the enzyme production was determined.

pH
The effect of pH on keratinase production was determined by culturing the fungus in the production media with different pH.

Inoculum level
Fermentation experiments were carried out using different levels of inoculums

Effect of agitation on enzyme production
Keratinase production in the cultures agitated and agitated at different rates was determined.

Supplementary carbon sources
The effect of different supplementary carbon sources on the keratinase production by *A.flavus*was studied using the media containing each of the different carbon sources as supplement were tested at a fixed concentration of 0.5% (w/v).

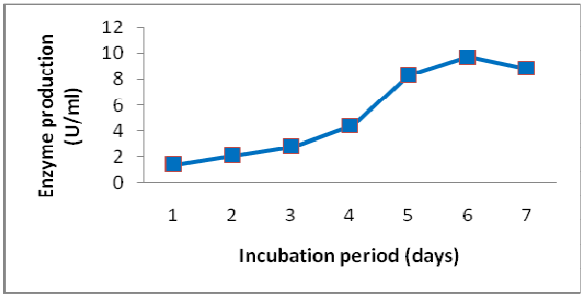
Supplementary nitrogen Sources
The effect of different supplementary nitrogen sources on the keratinase production by *A.flavus*was studied using the media containing each of the different nitrogen sources as supplement were tested at a fixed concentration of 0.5% (w/v).

Effect of surfactants
To identify the surfactants facilitating keratinase production, four different surfactants were used for experimentation. They were Tween-20, Tween-80, SDS (Sodium dodecyl sulphate) and PEG (Poly Ethylene Glycol). The surfactants were tested individually at the concentration of 0.2% in the optimized production medium.

RESULTS

Effect of incubation period
Keratinase production by the strain *A. flavus* at different incubation periods are shown in the figure 3.3

Figure 3.3
Effect of incubation period on keratinase production by Aspergillus flavus S125 bySmF

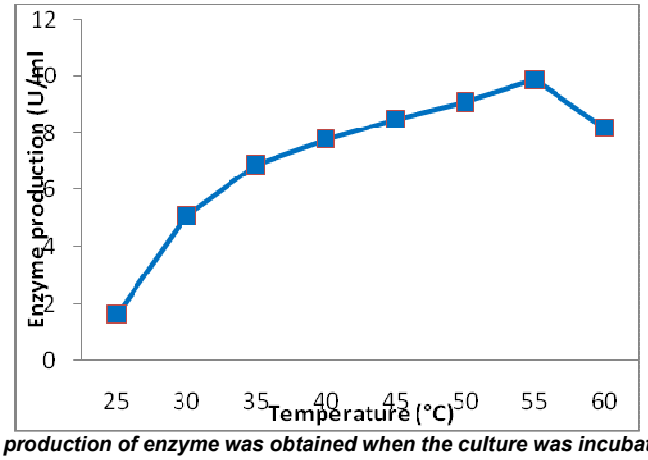


Maximum production of enzyme was obtained after 6 days of incubation by A.flavus S125 by SmF.

Incubation temperature

Effect of incubation temperature on keratinase enzyme production by *A.flavus* was shown in the figure.3.4

Figure 3.4
Effect of temperature on keratinase production by Aspergillus flavus by SmF S125

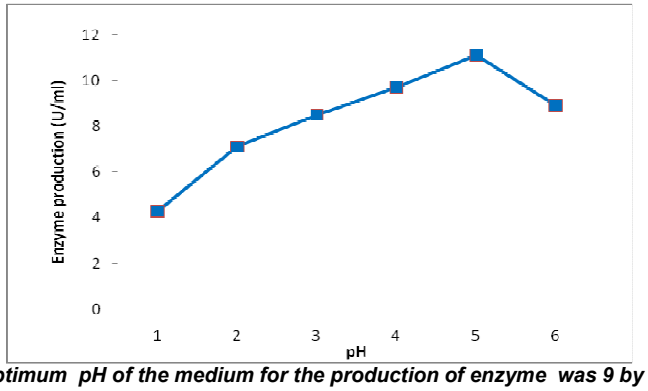


Maximum production of enzyme was obtained when the culture was incubated at 55˚C

pH

Effect of pH of the medium on the enzyme production is shown in the figure 3.5

Figure 3.5
Effect of pH on keratinase production by Aspergillus flavus by SmF S125



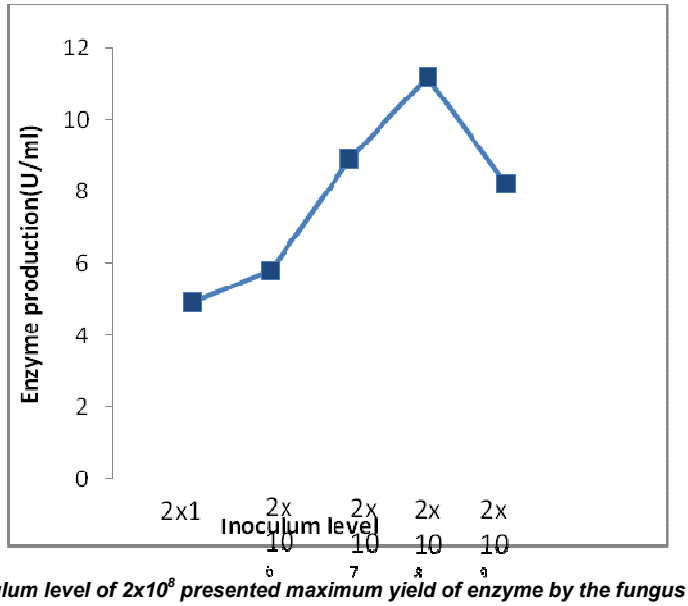
The optimum pH of the medium for the production of enzyme was 9 by SmF.

Inoculum level

The production of keratinase was found effected by different inoculum levels.

Figure 3.6

Effect of inoculum level on keratinase production by Aspergillus flavus by SmF

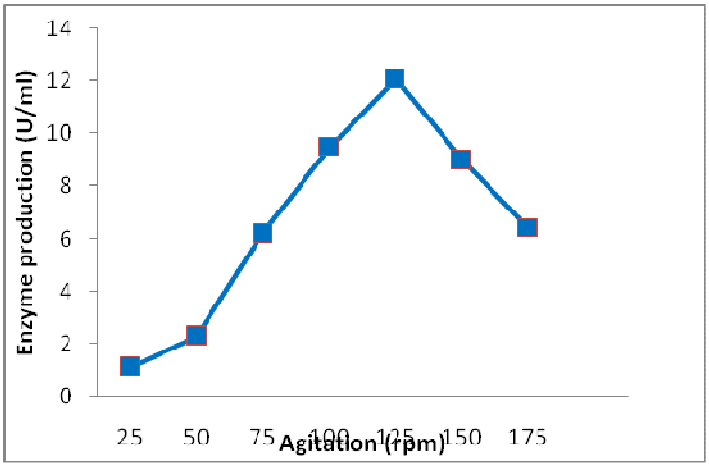


The inoculum level of $2x10^8$ presented maximum yield of enzyme by the fungus by SmF.

Effect of agitation

Figure 3.7

Effect of agitation on keratinase production by A.flavusS125 by SmF

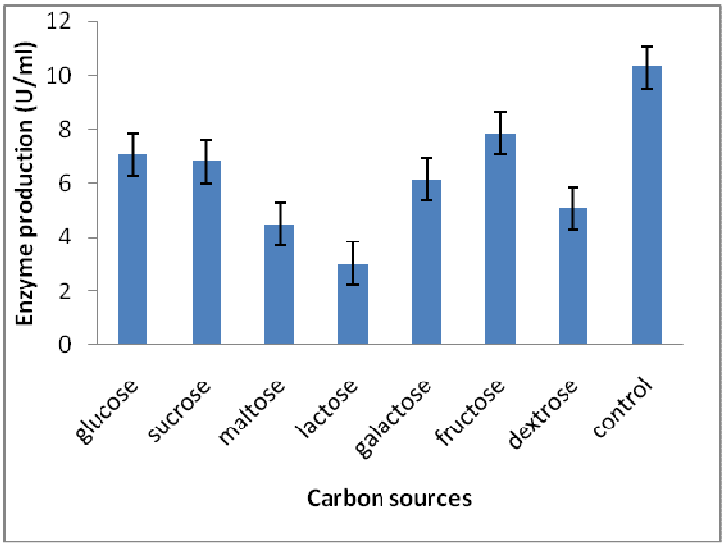


The culture showed an increase in enzyme production with the increase in agitation upto125 rpm and decrease in the production with further increase in agitation rate. Remarkable increase in keratinase production on agitation was obtained.

Effect of carbon sources

The effect of carbon sources in enzyme production by *A.flavus*S125 by SmF is shown in figure 3.8. Enzyme production was not found enhanced with any of the supplementary carbon sources used.

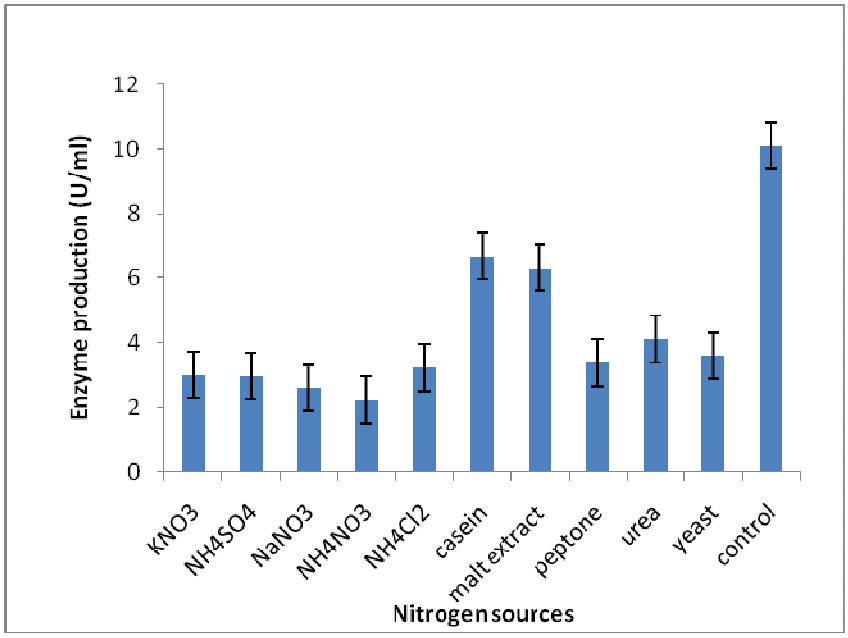
Figure 3.8
Effect of carbon sources on keratinase production by A.flavusS125 by SmF



Among the seven carbon sources used for the enzyme production none of them exceeded the control in the result.

Effect of Nitrogen sources
All the nitrogen sources were seen insignificant in enhancing the enzyme production.

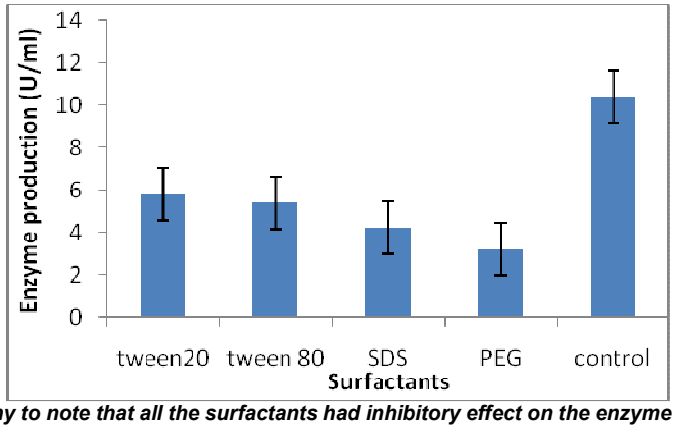
Figure 3.9
Effect of nitrogen sources on keratinase production by Aspergillus flavus by SmF



Presence of nitrogen sources had inhibitory action on the enzyme production. Significant decrease in enzyme yield was noted in most of the nitrogen sources.

Effect of surfactants

Figure 3.10
Effect of surfactants on keratinase production by A.flavus S125



It was worthy to note that all the surfactants had inhibitory effect on the enzyme production.

DISCUSSION

ptimization of culture conditions is essential to obtain maximum yield by the selected fungus.Various physical and chemical factors were found influencing the production of keratinase by *Aspergillus flavus* negatively or positively. A gradual increase in enzyme production was observed with the progress in the period of incubation. The maximum production was obtained at 6th day of incubation; thereafter the enzyme production was found reduced. Many investigators found that after 6 days of incubation, a drop in protease production was observed. [23]revealed that this phenomenon might be due to the decrease in microbial growth associated with the depletion of available nutrient, loss of moisture content, production of toxic metabolites, and degradation of produced protease. Different fermentation periods have been also reported by other workers for the best production of enzyme i.e. 9 days for *Rhizopusoryzae*[1]. The maximum enzyme production by the fungus *A. flavus* S125 was observed at 55˚C. Keratinase production by a newly isolated fungus *Myceliophthora*was found maximum at temperature 50˚C. [28]The optimal conditions for the keratinolytic activity were determined to be pH 9.0 and temperature 60°C in a *Bacillus* sp [29]Keratinolytic enzyme production was maximum at pH 9 and similar results were reported in the fungi *Doratomyces microspores*[9] and *Aspergillusflavus* [11]According to [13]dermatophytes and non dermatophytes metabolize free or combined cystein as a source of sulphur and nitrogen. Inorganic sulphur and other intermediates were the products of cystine metabolism by the fungi. It also indicated that the excessive sulphur is excreted back to the medium in the oxidized form as sulphate and sulphite. At a neutral to alkaline pH, sulphite reacts with cystine, cleaving it to cystein and S-sulphocystein. The parameters for keratinase production are species specific and vary with respect to the organism [7, 20, 26, 18, 24] It has been observed that alkaline pH supports keratinase production and feather degradation in most microorganisms. The optimum pH for *Streptomyces*sp SG-10 and *Streptomyces*sp Ktn-1 was found to be 9 and 10 respectively[8]. In the present study *A. flavus*cultured on simple medium supplemented with chicken feather, produced a protease mixture with high keratinolytic activity at high pH, and was very effective in feather degradation, suggesting its potential use in biotechnological processes involving keratin hydrolysis. Moreover, the serine-type keratinase produced by the strain was active over a wide range of pH values and temperature. Agitation of culture at 125 rpm resulted in maximum enzyme production. Agitation has beneficial effects like providing homogeneity throughout the fermentation

period, promotion of gas transfer and facilitation of heat exchange. Agitation of medium was found to be enhancing the enzyme production considerably by influencing the availability of nutrients as well as dissolved oxygen to the organism. Of the different carbon sources tested not even one presented promising result. Thus, the result is suggesting that sugars suppressed enzyme production of the fungus. The specificity of a particular carbon sources on enzyme production was repressive when various sugars were added to the medium. Sugars suppression of protease appears to be common among fungi[2]. Usually glucose has negative effects on microbial proteinase including keratinase production. For example, the keratinase produced by strain *Aspergillusfumigatus*[21]*moactinomycetuscandidus*[10] and *Stenotrophomonas sp.* D-1[26] were partially inhibited by glucose. Simple sugars have been reported to suppress the synthesis of keratinase in the fungus *Myrotheciumverrucaria* [17] The results of the present study indicate that carbon source could make the fungus not in a need to utilize keratin as carbon source. However[15] on *Chrysosporiuqueenslandicum* and[6] on *Chrysosporiumgeorgiae* noted that the keratinase enzyme was inducible by keratin and its production was stimulated by glucose. Present study revealed that supplement of not even one nitrogen source resulted in a better production of keratinase by *Aspergillusflavus* S 125 isolate S125. The effects of nitrogen sources on keratinase production also vary. Supplementation of yeast extract resulted in maximal keratinase production by *Stenotrophomonas* sp. D-I[26] The addition of nitrogen sources had no or depressive effect on keratinase production as well as solubilization of feather keratin. Different nitrogen sources, $NH_4H_2PO_4$, ammonium citrate ($(NH_4)_2C_6H_6O_7$), NH_4Cl and KNO_3 highly inhibited keratinase production and feather solubilization by *Alt. tenuissima* K2 and *A. nidulans* K7[19]. These data confirm the inducible nature of keratinase. [15] and [6] noted that the keratinase enzyme production was inhibited by ammonia. [21]reported that additional nitrate supported the mycelial growth, but it repressed keratinase production in *A. fumigatus*. However, most reports describe a partial or complete repressive effect of the supplementation of cultures with small nitrogen [15, 6 ,22]. However, the drastic reduction in the keratinase production is due, or at least overwhelmingly due to catabolite repression by the nitrogen sources[4]. On the contrary, when[25] and[9] used microorganisms directly for the biodegradation of different keratin containing wastes, less solubilization of keratinous wastes could be observed with the use of additional carbon and nitrogen sources since the microorganism itself consumes the released products. Microbial keratinases are reported as inducible and substrate specific[3] Various keratinous substrates like chicken feathers[6] feather meal [16]; wool[10] and bovine hair[11] have been used as inducer of keratinase. For instance the gene Ker A, which encodes keratinase in *B. licheniformis,* is expressed specifically for feather degradation[14]. Therefore the presence of feather keratin as sole carbon and nitrogen source in the culture medium may result in the expression of the keratinolytic protease.In the present study the addition of surfactants presented no significant increase in the keratinase production. However[18] have reported that addition of surfactants to feather broth cultured with keratinolytic bacterium *Chryseobacterium* sp. resulted in decrease in keratinase production.

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