EVALUATION OF DEGRADATIVE PRODUCTS OF FEATHER DEGRADATION BY *BACILLUS SP.*

P. Jeevana Lakshmi and V. V. Lakshmi*

Abstract— Assay of products of degradation is important to evaluate the economic value of the possible by-products of the fermentation process as well as to get a suggestive idea for the mechanism of degradation. Four native keratinase producing isolates identified as Bacillus sps. and producing < 10KU/ml were subjected to strain improvement and optimization of parameters of fermentation to yield 500KU/ml. The four improved isolates (MBF11, MBF20, MBF21, MBF45) were analysed for degradative products of fermentation. The results clearly indicated a significant increase in the amounts sulfur containing amino acids with optimum levels observed on 5th to 6th days of fermentation. Maximum concentration cysteine was in the range of 12.5-14µg/ml whereas that of cystine reached 18.1-15.7 µg/ml for all the isolates. Maximum concentration of methionine reached 2.1-2.9 µg/ml. Quantity of inorganic sulphur is significantly lower than organic sulphur. Highest concentration, 3.1-3.7 µg/ml of free aminoacids was recorded for the four improved isolates. Sulfitolysis along with cleavage at other aminoacyl sites is the suggestive mechanism of degradation.

Index Terms— A mino acids, Bacillus, Feather Degradation, Keratinase

1 INTRODUCTION

Keratinases can play a vital role in recycling of poultry waste, which is hitherto a underutilized and limitedly trapped source of protein [1], [2]. The most prominent way to utilize keratin is as food and feed supplement. Keratinase treated feather and feather meal produced by biological treatment are emerging as viable alternatives for those produced by conventional methods in the last decade due to their better digestibility and significant improvement in nutritional content [3],[4], [5]. Though till recently feather has not been considered as serious source of dietary protein because of its poor digestibility, with the development of keratinase formulation, lot of interest is regenerated to use keratinase treated feather as economical animal feed supplement [6]. The keratinase treatment results in hydrolysis of keratin protein into short peptides and amino acids that are easily digestible. Further it can be used as a rich source for extraction of commercially important amino acids. Keratinases also have application in leather industry in addition to array of other application potentials. Hence, world wide attention is focused on developing keratinase technology by investigating for commercially viable isolates as well as improves their yields. In spite of several organisms being characterized worldwide [3], very few indigenous isolates of importance are reported from India the present study investigates the production of commercially important amino acids as by- products of feather degradation.

- *P. Jeevana Lakshmi, Department of Applied Microbiology, Sri Padmavati Mahila Visvavidyalayam, Tirupati- 517502*
- *V. V. Lakshmi,Professor, Department of Applied Microbiology, Sri Padmavati Mahila Visvavidyalayam, Tirupati- 517502*

2 MATERIALS AND METHODS:

Four potentially important isolates BF11, BF20, BF21 and BF45 were developed to yield high keratinase activity by subjecting to strain improvement. The application potential of the improved isolates MBF11, MBF20, MBF21 and MBF45 grown in optimized media were containing feather studied was investigated for commercially important by products produced on degradation of feather. Fermentation is conducted in 250ml flasks with 1% feather. The samples were collected at 24hour intervals for a period of 7 days where complete degradation of feather was achieved and analyzed for by-products.

2.1 Assay of degradative products:

Study of the by-products profile of any fermentative process is important to assess the application potential of the enzyme and in improving the economic value of the products. Degradative products of keratinase fermentation like sulphur rich compounds cysteine, cystine, total inorganic sulphur released and amount of free amino acids were estimated for all the four isolates.

The cysteine produced by degradation of feather was assayed by adopting the method of Ramakrishna et al. [7]. This method was prepared as it was found to be sensitive in quantitating cysteine even in the presence of other naturally occurring amino acids. Standard cysteine solution was taken in the concentration range of 0.053mg to 0.368mg and a sample without cysteine was maintained as blank. The colour developed was measured at 510nm in UV-visible spectrophotometer.

The assay of cystine was done using the method of Ramakrishna et al., [7] by hydrolyzing cystine to cysteine and then assaying the cysteine spectrophotometrically as described above. Standard cystine (24mg/100ml) solution was taken in the concentration range of 0.24mg – 2.4mg per ml. The hydrolyzed and unhydrolyzed samples were compared to calculate

the amount of cystine in the samples.

The amount of methionine released during keratinase production was estimated by adopting the method of [8]. The O.D of the samples was read at 530nm. The readings were corrected by considering the absorption values of the reagent blank (containing all ingredients except nitroprusside) and a blank containing all ingredients except methionine standard.

Increase in the free amino groups released by proteolytic degradation of the keratin substrate during the process of fermentation was estimated with the modified Ninhydrin method of [9]. Leucine in the concentration range of 0.02mg to 0.2mg was taken as standard for creating standard curve. The O.D was recorded at 520nm.

Turbidometric method for the micro-determination of sulfur in proteins was adopted to assay inorganic sulfate content in the culture filtrates [10]. Potassium sulphate was taken as standard solution in the range of 0.87mg to 17.4mg. The concentration of inorganic sulphur was determined by measuring the developed turbidity at 490nm spectrophotometrically.

3 RESULTS AND DISCUSSION:

The degradative products produced during the process of fermentation were estimated to ascertain the application potential of the by-products produced during biodegradation of feather. The type and quantity of degradation products produced also give a suggestive idea for the mechanism of degradation. The analysis of culture filtrate showed a significant increase in the amounts of cysteine, cystine, methionine, and total free amino acids during the fermentation period (Tables 1 and 2, Fig.1). There was a gradual increase in concentration of cysteine from 2.5µg/ml on first day to 12.5-14µg/ml by fifth day for MBF11, MBF20, MBF21 and MBF45. Higher levels of cysteine (6.7µg/ml) were detected from the first day itself from MBF45 and continued to increase till fifth day after which the trend declined. Higher cystine levels were detected in the culture filtrate of MBF21 and MBF45 as compared to MBF11 and MBF20 from the first day. The cystine levels reached peak by fifth day for all the isolates after which a slight decline was observed. The amount of methionine detected was significantly lower as compared to cysteine and cystine with all the MBF isolates. The concentration of free amino acid was <0.4 - 0.8mg/ml on first day which gradually increased to 12-15mg/ml by fifth day where maximum degradation of substrate was observed. The content followed a decline trend by sixth to seventh day as the degradation of substrate reached almost complete. The results thus indicate that the increase in the concentration of cysteine and cystine as well as total free amino acids correlated well with the levels of degradation of the substrate. The levels of soluble amino acids like cysteine and cystine released by the MBF isolates were comparable or higher than earlier reports where 5µg to 16µg/ml was observed in different studies [11], [12]. Though, 35µg/ml cystine was reported to be released on degradation of hair by Microsporum gypseum by [13], the period of incubation was 50 days.

The mechanism of keratinolysis is highly complex and has been investigated in fungi, actinomycetes and bacillus to understand the process [3], [14], [15], [16]. Studies with different microorganisms suggest that proteolytic attack along with mechanical keratinolysis account for degradation of keratin. The keratin degradation in several cases is observed to commence even before the detection of significant extracellular keratinase. Hence, mechanical keratinolysis was considered to be an integral part of the mechanism for keratin degradation by filamentous microorganisms and a synergism between mechanical and enzymatic hydrolysis is suggested for keratin digestion[13], [17] Similar pattern of degradation was observed in the current study and it was partial in first 2-3 days (mechanical) followed by keratinase degradation of feather i.e., total by seventh day.

Table 1: Analysis of degradative products in culture filtrate of MBF11 and MBF20

Isolate	By-product of fermentation	Fermentation period (Days)						
		1	2	3	4	5	6	7
MBF11	Cysteine (µg/ml)	2.5	7.5	13.5	13.8	14.0	13.2	12.0
	Cystine (µg/ml)	2.8	3.2	3.9	7.7	12.0	12.8	7.8
	Methionine (µg/ml)	1.2	1.8	2.2	2.6	2.8	2.9	2.1
MBF20	Cysteine (µg/ml)	2.7	7.5	10.5	11.5	13.0	11.7	8.5
	Cystine (µg/ml)	3.7	5.7	9.4	9.7	11.5	9.1	8.9
	Methionine (µg/ml)	0.7	1.3	1.8	2.1	2.4	1.8	1.6

Table 2: Analysis of degradative products in culture filtrate of MBF21 and MBF45

Isolate	By-product of fermentation	Days of fermentation						
		1	2	3	4	5	6	7
MBF21	Cysteine (µg/ml)	2.5	8.5	9.0	12.0	12.5	11.7	10.5
	Cystine (µg/ml)	7.3	9.3	12.1	12.6	18.1	17.9	10.8
	Methionine (µg/ml)	0.9	1.3	1.6	1.9	2.1	2.0	1.6
MBF45	Cysteine (µg/ml)	6.7	7.0	11.2	12.5	13.5	12.5	12.0
	Cystine (µg/ml)	5.1	6.0	8.1	11.8	15.7	14.8	10.6
	Methionine (µg/ml)	1.4	1.9	2.2	2.6	2.9	2.2	1.8

The inorganic sulphate concentration was significantly lower than the organic sulphur in the culture filtrate for all

the MBF isolates. With a number of filamentous fungi, thiosulphate was the major by-product released followed by cysteine, cystine. Release of thiol groups was largely accounted by reduction in disulfide bonds by enzymatic disulphide reduction [18] , [19], [20]. The amount of inorganic sulphur, peptides / amino acids or other sulphohydrl compounds liberated from degradation are considered as index of degree of keratinolytic activity of an organism [21] , [22]. The detection of the products of sulphitolysis such as peptides, cysteine, cysteine, sulphate in the culture filtrate of MBF isolates confirmed the disulphide breakdown. Further the ratio of sulphur compounds released by MBF isolates showed that the inorganic sulphate concentration was significantly lower than the organic sulphur in the culture filtrate for all the isolates. Further 70% of the total organic degradative sulphur products released were constituted by Sulphur containing amino acids released between 96-120 hours of fermentation indicating the economic importance of the keratinase as well as byproducts produced.

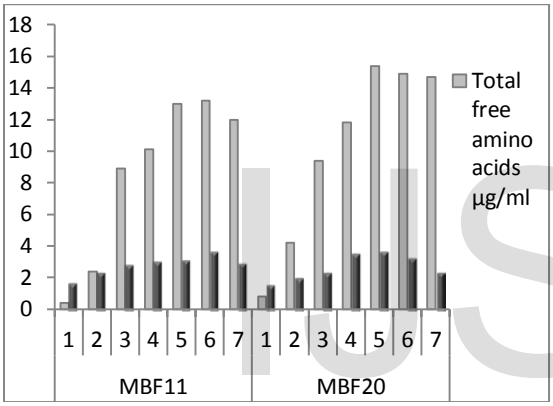

Figure 1: Analysis of total free amino acids and inorganic sulphur in the culture filtrate

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