Fermentative production of keratinase using solid agricultural wastes

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Abstract— Keratinases are robust and have high potential to degrade keratin wastes. Present study is oriented towards utilization of solid agricultural wastes as fermentation substrates by the *Bacillus sps* offers development of a low-cost medium for producing keratinase enzyme. To economize media cost for production of keratinase further, in the present study agricultural byproduct waste like wheat bran, rice bran, green gram husk, black gram husk were tested as nitrogen sources to replace SM/GC. Optimization of parameters for fermentative production of keratinase enzyme in our earlier studies resulted in design of media with starch as carbon source and soyabean meal/ ground nut cake as nitrogen source. Keratinase production was highest with rice bran for MBF11 with maximum activity of 318KU/ml, black gram for MBF20 (354KU/ml), green gram for MBF21 (374KU/ml) and wheat bran for MBF45 respectively (497KU/ml). Hence solid agricultural wastes have the possibility of replacing fermentation substrates that were used in earlier studies which offers development of a low-cost microbial technology for producing keratinase enzyme and it is also eco-friendly.

Index Terms— Keratinase, low-cost medium ,solid agricultural waste, fermentation, black gram, green gram, wheat bran, rice bran, nitrogen source

1 INTRODUCTION

Keratinases mainly belong to Serine/ Metallo protease groups and are mostly extra cellular and inducible enzymes. Feather is resistant to degradation by common microbial proteases like trypsin, pepsin and papain *etc.* [1] Effective recycling and utilization of this grossly underutilized waste is of great economic and ecological importance. Slow recycling of feather in nature results in accumulation of feather dumps leading to pollution. Thus inspite of being protein rich, this by-product is under utilized and is thus wasted. Hence, bioremediation of this high voluminous waste has gained immense prominence. Despite being recalcitrance to common proteases, keratin is found to be attacked by keratinases produced by microorganisms and degraded in nature.

Enzyme processing being superior to conventional methods has increasingly employed in today's high technological society. Their specificity, selection to particular reaction and regio-specificity yields relatively uncontaminated end products makes them eco-friendly. Production of microbial enzymes on large scale can be carried out by submerged or surface culture methods using liquid or solid media. Fermentative production of majority of products on industrial scale over the years has been dominated by submerged culture method. The method had preference because of low handling costs, higher yields, less risk of contaminating organisms, uniform control of parameters of fermentation like temperature, humidity, aeration *etc*. The process is especially useful in production of microbial enzymes from agricultural waste materials as substrates [2]. The vast application potential for keratinase has resulted in a drive for production of keratinase by fermentation at industrial scale. Knowledge of fermentation parameters, purification strategies and properties of the bio-molecules is essential to develop efficient enzyme based production process. The process adopted ideally should require minimum time and investment and efficient recovery rate. Keratinase has been produced successfully by adopting submerged fermentation technique. The enzyme was also found efficient in converting feathers into poultry feed supplement [3] and other potential applications.

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2 MATERIALS AND METHODS

In the fermentation process starch and soyabean meal/ groundnut meal were found to be optimum carbon and nitro-

gen sources for keratinase production for MBF isolates of *Bacillus* species in our earlier studies. Utilization of solid agricultural waste products like wheat bran, rice bran, green gram husk, black gram husk can serve as other cheaper nitrogen sources for fermentation.

2.1 Use of agricultural byproducts in design of media

Present fermentation process media was designed with solid agricultural byproducts at 1% concentration were substituted instead of nitrogen source. In starch production medium (SPM) WB, RB, GG and BG was used. 1% soyabean meal was used as control for MBF11 & MBF20 whereas 1% groundnut meal for MBF21 and MBF45. Starch production medium along with agricultural wastes was sterilized by using autoclave at 15lb/inch^2 for 15 minutes. The medium was inoculated with 5ml of 12 hour old inoculum (~10^5 CFU/ml) of the respective culture. The fermentation process was carried out for 7 days and keratinase enzyme activity was determined at intervals of 24 hours as described below. .

2.2 Keratinase assay

The assay of keratinase activity was carried out by adopting the method of Lin *et al*. [4] 10mg of azokeratin was taken in a 5ml test tube and 1.6ml of 50mM potassium phosphate buffer (pH-7.5) was added. The mixture was agitated until the azokeratin was completely suspended. 0.4ml of an appropriately diluted enzyme sample was added to this mixture and mixed thoroughly. The sample was incubated for 15 minutes at 50°C. The enzyme reaction was terminated by adding 0.4ml of 10% Trichloroacetic acid (TCA). The reaction mixture was filtered through Whatman's No.1 filter paper and analyzed for activity. The absorbance of the filtrate was measured at 450nm with a UV visible spectrophotometer (Systronics - 117). Appropriate control samples were prepared for each sample analyzed by adding the TCA to the reaction mixture before the addition of enzyme. Unit of keratinase activity in the assay was defined as 0.01 increase in the absorbance at 450nm as compared to the control after 15 minutes of reaction.

3 RESULTS AND DISCUSSIONS

To explore economic nitrogen sources, wheat bran(WB), rice bran (RB), green gram husk (GG), and black gram husk(BG) were tested as nitrogen source along with starch as carbon source in starch production medium (SPM). For development fermentation the nitrogen source soyabean meal (SM) was taken as control for MBF11, MBF20 and groundnut cake (GC) for MBF21, MBF45. The results are shown in Tables 1 - 4. MBF11 showed increased keratinase production with RB as substrate compared to the control SM which had activity of 300KU/ml. A maximum keratinase activity of 318KU/ml was observed with RB supplementation followed by WB (210KU/ml), BG (144KU/ml) and GG (105KU/ml). MBF20 on the other hand showed highest keratinase activity with BG (354 KU/ml) followed by GG (329KU/ml). These were comparable with SPM supplemented with SM which was taken as control. Enzyme activities for other substrates like WB and RB was similar marginal though lower (240KU/ml). With MBF21 also, Keratinase activity was highest with GG (375KU/ml) followed by WB (224KU/ml), RB (156KU/ml) and BG (130KU/ml) supplementation. Green gram was found to exhibit higher keratinase yield compared to control for both MBF20 and 21. MBF45, however showed maximum keratinase yield of 498KU/ml with WB followed by BG (476KU/ml), RB (418KU/ml), GG (355KU/ml) respectively. Keratinase activity for SPM with groundnut cake taken as control was 384 KU/ml.

Table 1: Nitrogen source for keratinase production by MBF11

Agricultural wastes	Fermentation period (Days)						
	1	2	3	4	5	6	7
	Keratinase activity (KU/ml)						
SPM+WB	78	138	169	196	204	**210**	179
SPM+RB	75	79	286	**318**	299	279	233
SPM+GG	83	93	98	103	**105**	100	98
SPM+BG	68	79	128	**144**	100	104	100
SPM+SM	82	141	189	246	**300**	230	206

Table 2: Nitrogen source for keratinase production by MBF20

Agricultural wastes	Fermentation period (Days)						
	1	2	3	4	5	6	7
	Keratinase activity (KU/ml)						
SPM+WB	71	123	155	228	**240**	108	124
SPM+RB	86	134	232	**240**	198	132	101
SPM+GG	20	131	236	**329**	235	136	129
SPM+BG	10	158	247	**354**	239	143	131
SPM+SM	121	173	236	233	253	275	**349**

Fig. 1 illustrates the comparison of the various nitrogen sources on keratinase production along with the control for the MBF strains. The results indicate that keratinase activity was highest with rice bran for MBF11 where as it was maximum with black gram for MBF20. Green gram was found to support optimum keratinase yield with MBF21 whereas keratinase activity was highest with wheat bran for MBF45.

Commercial exploitation of any fermentative product requires that the product be produced in high yields to reduce the cost of the end product. The viability of the enzyme production depends to a large extent on the media used, duration

of fermentation and downstream operations required for purification and extraction of enzyme. Triangular interactions between culture improvement, development of media and optimization of process conditions are crucial, and have been importantly recognized in production of many enzymes. Any improvement made in any one of these areas can lead to several opportunities in the other two field areas also [5],[6],[7]. Cost and availability of components of media being major factors, sign of a fermentation media, bulk agricultural byproducts or other relatively cheap carbon and nitrogen sources such as starch, molasses, soyabean meal, groundnut cake agricultural waste *etc.*, are preferred over reagent grade or pure chemicals as media components [8],[9],[10][11]. As economics play vital role in overall efficiency of the process, the incorporation of relatively inexpensive raw materials in the fermentation medium, greatly bring down the overall cost for scaling up of production [12],[13]. Hence, it was important to determine optimum carbon and nitrogen sources and C/N ratio supporting maximum keratinase production thus making it cost effective process.

Table 3: Nitrogen source for keratinase production by MBF21

Agricultural wastes	**Fermentation period (Days)**						
	1	*2*	*3*	*4*	*5*	*6*	*7*
	Keratinase activity (KU/ml)						
SPM+WB	*161*	*173*	*182*	*175*	*224*	*146*	*130*
SPM+RB	*69*	*78*	*129*	*156*	*100*	*128*	*103*
SPM+GG	*203*	*278*	*296*	*375*	*303*	*288*	*285*
SPM+BG	*83*	*87*	*92*	*82*	*88*	*130*	*103*
SPM+GC	*215*	*222*	*241*	*321*	*209*	*199*	*140*

Table 4: Nitrogen source for keratinase production by MBF45

Agricultural wastes	**Fermentation period (Days)**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
	Keratinase activity (KU/ml)						
SPM+WB	18	121	220	**498**	322	209	160
SPM+RB	10	120	223	**418**	224	196	150
SPM+GG	24	125	228	**355**	239	128	121
SPM+BG	20	131	221	**476**	325	229	119
SPM+GC	163	168	304	349	200	**384**	170

Optimization of parameters for fermentative production of keratinase enzyme in our earlier studies resulted in design of media with starch as carbon source and soyabean meal/ ground nut cake as nitrogen source [14]. To economize media cost for production of keratinase further, in the present study agricultural byproduct waste like wheat bran, rice bran, green gram husk, black gram husk were tested as nitrogen source to replace SM/GC in SmF. Keratinase production was highest with rice bran for MBF11 with maximum activity of 318KU/ml, black gram for MBF20 (354KU/ml), green gram for MBF21 (374KU/ml) and wheat bran for MBF45 respectively (497KU/ml) as observed from Tables 1 - 4. In earlier studies SM was found to be optimum nitrogen source of MBF11 and MBF20 from among several organic and inorganic nitrogen sources tested. Similarly GC was found to be optimum for MBF21 and MBF45 [14]. These were taken as controls to compare keratinase yields by the test strains. The yield of keratinase with SM was 300KU/ml with MBF11 and 349KU/ml for MBF20. Similarly keratinase yield with GC was 321KU/ml with MBF21 and 384KU/ml for MBF45. The results thus suggest that the production of keratinase was comparable or higher between the optimum bran agricultural byproduct identified and SM/GC, both in terms of maximum yield as well as extent of degradation of feather. Thus agricultural byproducts can be equally effective as nutrient source thus economizing media cost further.

The choice of nitrogen and carbon sources has been shown to have a major influence on the yield of enzymes. Different bacteria have varied preferences for either organic or inorganic nitrogen for growth and enzyme production. Complex nitrogen sources are preferentially utilized for alkaline protease production in several earlier studies [15],[16].. The findings from our studies also confirm the fact that MBF strains tested in the present study showed preference for complex organic nitrogen sources and brans over inorganic nitrogen for keratinase production. Peptone and yeast extract were however, found to suppress the protease production in some cases like alkaliphilic strain of *Arthrobacter ramosus* MCM B351 which was similar to our earlier observation [17],[18]. Complex organic nitrogen sources have also been reported to enhance the secretion of exoprotease in *B. licheniformis* strain. Soyabean meal was found to be the best among numerous nitrogen sources tested [19],[20]. Similarly protein rich media have been found to induce keratinase production in *B.licheniformis* and *B.subtilis* FBD-29 strains as well as MBF Bacillus strains in our earlier studies also [21],[22]. Keratinase production increased up to 2-fold with increase in the organic nitrogen and carbon sources from 0.2 to 1% after that a plateau observed for the isolates according to Chen *et al.,* [23]

Use of complex by-products of agriculture origin as nitrogen source, can make the bioremediation process rapid along with efficiently controlling foaming during fermenta-

tion. In addition these bio-products can act as pH regulators also [24]. In view of the lower cost of these products as well as regional availability, use of these agricultural byproducts can have significantly positive impact in economizing the overall cost of the media.

Our present study also confirmed our earlier observation that keratinase production was enhanced when starch was supplied as co-carbon source to the medium. This observation is in accordance with that of study of Prakash *et al.* 2010 and several others who observed no repressive effect on enzyme production by *Bacillus* sp. with additional carbon source. However, catabolic repression has been observed in case of several proteases as well as keratinases [25]. Chen *et al.* [23] described complete inhibition of the extracellular protease production from *Geobacillus caldoproteolyticus strain* SF03 in presence of glucose, a versatile carbon source.

Enhancement in production of enzymes was observed in earlier studies both in *Bacillus* sp. as well as in *Streptomyces* sp. *w*hen agro-industrial waste was used. About 20% increase in protease enzyme synthesis was observed when wheat bran was supplemented with peptone in case of *Bacillus* sp. AR-009 [26]. Supplementation of wheat bran with corn-steep liquor also significantly increased xylanase activity with *Bacillus licheniformis* [27]. Appreciable levels of difference in xylanase and other proteases yields was observed with and without supplementation of wheat bran, suggesting nutrients contributed by bran could better support growth of the microorganisms as well as their product production [28],[29]. *Penicillium* sp. Morsy1 was able to produce keratinase using rice straw as substrate [30].

4 CONCLUSIONS

The present study intended to explore the application potential of biological processed feather by using keratinase. The results of our current study clearly suggest that optimum keratinase production by MBF strains was supported by bran as nutrient source at optimised physical parameters. Although solid substrates have been used for the production of bacterial alkaline proteases, use of agricultural wastes for keratinase production by *Bacillus* sp. has been reported here for the first time. Utilization of agricultural wastes as fermentation substrates by these micro organisms offers development of a low-cost microbial technology for producing keratinase enzyme which is also eco-friendly.

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6 REFERENCES

[1] M.Holt, and F.Sanders," Fermentation process development of industrial organisms", Recent advances in the Molecular Biology. B.Justin.O.Neway,-Science. 21:pp. 324. 1986.

[2] A.Pandey," Solid-state fermentation",*Biochemical Engineering Journal* 13:pp. 81-84, 2003.

[3] S. S. Nilegoankar, P. P.Kanekar, S. S. Sarnaik, and A. S.Kelkar," Production, isolation and characterization of extracellular protease of an alkaliphilic strain of Arthrobacter ramosus MCM B-351 isolated from the alkaline lake of Lonar, India", World Journal of Microbiology and Biotechnology, 18: pp. 785-789, 2002.

[4] X.Lin, C. G.Lee, E. S.Casale, and J. C. H.Shih, "Purification and characterization of a keratinase from a feather-degrading Bacillus licheniformis strain", Applied and Environmental Microbiology, 58: pp.3271-3275, 1992.

[5] P.Jeevana Lakshmi, Ch. M. Kumari Chitturi and V. V. Lakshmi, "Purification and Characterization of Keratinase from Feather Degrading Bacillus sp", The Internet Journal of Microbiology. ISSN: 1937-8289, 2013.

[6] A.Crueger, Biotechnology," A text book of Industrial Microbiology. 2nd edition. Panima. New Delhi", pp. 47-58. 2000.

[7] N.Sangeetha, and B.Rintu," Optimization of culture parameters to enhance production of amylase and protease from Aspergillus awamori in a single fermentation", African Journal of Biochemistry Research. 4: pp. 73-80. 2010.

[8] C.Alena, "Preparation of media. In Biotechnology," eds Rehm, H.-J. and Reed, G. Weinheim: VCH. 2: pp. 631-698, 1985.

[9] K.Corbett," Design, preparation and sterilization of fermentation media. In: Comprehensive biotechnology" Moo-Young M (ed) Pergamon Press, NY, pp. 127–139, 1985.

[10] P. F.,Stanbury, S.Hall, and Whitaker,"Principles of Fermentation Technology", Second Edition: Publisher: Butterworth-Heinemann. 1999.

[11] Nereida Mello Rosa da Gioppo, G. Fabiana Moreira-Gasparin, Andréa M. Costa, Ana Maria Alexandrino and Cristina Giatti Marques de Souza, "Influence of the carbon and nitrogen sources on keratinase production by Myrothecium verrucaria in submerged and solid state cultures", Journal of Industrial Microbiology and Biotechnology, 31: pp. 705-711, 2009.

[12] L. E.Casida, Industrial Microbiology. New Age International, New Delhi. pp. 34-58, 1968.

[13] A.Crueger," Biotechnology: A text book of Industrial Microbiology", 2nd edition, Panima, New Delhi. 47-58. 2000.

[14] P.Jeevana Lakshmi, "Fermentative production of keratinase by Bacillus sp. And its relevance to recycling of poultry feather waste", Ph.D Thesis submitted to Sri Padmavathi Mahila Visvavidyalayam, Tirupati. 2007.

[15] A.Pandey," Solid-state fermentation", *Biochemical Engineering Journal.* 13: pp.81-84. 2003.

[16] A.Pandey, C. R. Soccol and D. Mitchell, "New developments in solid state fermentation :I bioprocess and products", Process Biochemistry. 35: pp.1153-1169, 2000.

[17] S. S.,Nilegoankar, P. P.Kanekar, S. S. Sarnaik, and A. S. Kelkar, "Production, isolation and characterization of extracellular protease of an alkaliphilic strain of Arthrobacter ramosus MCM B-351 isolated from the alkaline lake of Lonar, India", World Journal of Microbiology and Biotechnology, 18:pp. 785-789, 2002.

[18] M.Holt, and F.Sanders," Fermentation process development of industrial organisms" Recent advances in the Molecular Biology. B.Justin.O.Neway.1989-Science. 21: pp.324. 1986.

[19] M.Srinivasan and S. C. Dhar," Effect of carbon and nitrogen on exo-protease synthesis in batch cultures of Bacillus licheniformis NCIM 2042", ndian Journal of Experimental Biology, 26: pp.22–24, 1988.

[20] P.Jeevana Lakshmi, "Fermentative production of keratinase by Bacillus sp. And its relevance to recycling of poultry feather waste",Ph.D Thesis submitted to Sri Padmavathi Mahila Visvavidyalayam, Tirupati, 2007.

[21] L. E.Casida, "Industrial Microbiology. New Age International, New Delhi", Pp. 34-58, 1968.

[22] R.,Chakraborty, M.,Srinivasan, S. K.Sarkar, and K. V.Raghavan, "Production of acid protease by a new Aspergillus niger by solid state fermentation",Journal of Microbiology and Biotechnology, 10:pp. 17–30, 1995.

[23] X-G.,Chen, O.Stabnikova, Joo-Hwa Tay Jing-Yuan Wang Æ Stephen Tiong-Lee Tay, "Thermoactive extracellular proteases of Geobacillus caldoproteolyticus, sp. nov., from sewage sludge", Extremophiles, 8: pp.489–498, 2004.

[24] L. E.Casida, "Industrial Microbiology", New Age International, New Delhi.pp. 34-58, 1968.

[25] K.Corbett, "Design, preparation and sterilization of fermentation media" In: Comprehensive biotechnology. Moo-Young M (ed) Pergamon Press, NY, pp.127–139, 1985.

[26] A.Gessesse, and G.Mamo, "High-level xylanase production by an alkaliphilic Bacillus sp. by using solid-state fermentation", Enzyme Microbial Technology, 25: pp. 68–72, 1999.

[27] A.Archana and T.Satyanarayana, "Xylanase production by thermophilic Bacillus licheniformis A99 in solid-state fermentation", Enzyme Microbial Technology, 21: pp.12–17, 1997.

[28] Sara Solis-Pereira, Ernesto Favela-Torres, Gustavo Viniegra-González and Mariano Gutiérrez-Rojas,"Effects of different carbon sources on the synthesis of pectinase by Aspergillus niger in submerged and solid state fermentations", Applied Microbiology and Biotechnology, 39: pp.36-40, 1991.

[29] K. R.Babu and T. Satyanarayana, "Amylase production by thermophilic Bacilluscoagulans in solid state fermentation", Process Biochemistry. 30: pp.305-309, 1995.

[30] A.Mervat Morsy El-Gendy,"Keratinase Production by Endophytic Penicillium spp. Morsy1 Under Solid-State Fermentation Using Rice Straw", Applied Biochemistry Biotechnology, 162: pp.780–794, 2010.

Fig. 1: Comparison of supplementation of various nitrogen

sources on keratinase production