European Journal of Biotechnology and Bioscience
Online ISSN: 2321-9122
www.biosciencejournals.com
Received: 01-01-2016: Accepted: 02-02-2016
Volume 4; Issue 2; February 2016; Page No. 01-04

Development of semi-solid state fermentation of Keratinase and optimization of process by cheaper and alternative agricultural wastes

Ch. M kumari Chitturi, * VV Lakshmi

Department of Applied Microbiology, Sri Padmavathi Mahila Visvavidyalayam, Tirupati-517502, India

Abstract
Keratinases are largely serine or metallo proteases. They attack the keratin residues and find application in developing cost-effective feather by-products for feed, fertilizers, detergent and leather industries. In the present study semi SSF method for keratinase production using *Bacillus* sp. was developed that appears to be a viable and economic alternative of bioconversion of large quantities of feather. Keratinase production was highest with rice bran for MBF11 (318KU/ml), black gram for MBF20 (354KU/ml), green gram for MBF21 (374KU/ml), wheat bran for MBF45 (497KU/ml). Standardization of moisture content resulted in identifying optimum conditions of SSF with only 33% of the original moisture content. Keratinase yield was found to be higher in SSF as compared to SmF for MBF strains. Substrate concentration was found to influence keratinase yield between 1-4% for the four *Bacillus* strains with yields increasing from 283-367KU/ml to 415-442KU/ml.

Keywords: solid state fermentation, *Bacilli*, cost-effective feather by-products, Keratinase activity, agricultural waste materials

Introduction
The vast application potential for keratinase has resulted in a drive for production of keratinase by fermentation at industrial scale. Keratinase has been produced successfully by adopting submerged culture technique. Semi SSF offers numerous advantages over submerged fermentation system like simple fermentation equipment, high volumetric productivity, reduced capital investment, cheaper cost of production, enzyme with better physiochemical properties and stability, less risk of contaminating organisms. Significantly less effluent are generated in addition to lower energy requirements. Agro-industrial residues are generally considered the best substrate for the SSF processes. A number of such substrates have been employed for the cultivation of microorganisms to produce products of biotechnological importance [1]. These include sugarcane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, grapevine trimmings dust, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch *etc*.
Feather degrading BF11, BF20, BF21 and BF 45 were isolated and characterized as *Bacillus licheniformis* and *Bacillus cereus* respectively. A significant 50fold increase in Keratinase yield was achieved in MBF isolates by strain improvement compared to native isolates. A cost effective fermentation media with starch as carbon source and soya bean meal as nitrogen source was designed along with optimization of physical parameters of fermentation resulting in a yield of >500KU/ml [2]. The present study aim is to develop solid state fermentation using solid agricultural waste materials to reduce the cost of fermentation for bioconversion of feather which is having several applications.

Materials and Methods
Keratinase applications mainly involve its use in crude form in degradation of keratinous material, it is of special interest or particular to check if the production of keratinase could be optimized by SSF. Keratinase activity was highest with rice bran (RB) for MBF11, with black gram husk (BG) for MBF20. Green gram husk (GG) was identified to exhibit highest keratinase yield for MBF21 and wheat bran (WB) for MBF45. These suitable agricultural wastes were supplemented in starch production medium for developing SSF.
Moisture content in starch production medium was reduced to standardize the SSF. The volume of water in the production media was reduced from 90ml original to 70ml (77%), 50ml (55%), 30ml (33%), 20ml (22%) respectively thereby reducing moisture content upto the range of 22-77% of that present in original submerged condition. Media was inoculated and incubated at 37˚C with shaking and samples were assayed at regular intervals for keratinase activity till 7 days. Medium containing 90ml (100%) moisture was taken as control for comparison. Nitrogen source tested above showing high keratinase yield were selected for each organism to standardize SSF. RB, BG, GG and WB were used for MBF11, MBF20, MBF21 and MBF45 respectively. Effect of feather substrate concentration on production of keratinase for MBF isolates by SSF was studied in the media containing optimized carbon and nitrogen source. The substrate concentration was varied between 1-4%.

Results
Moisture content in the medium is a significant factor influencing the production of enzyme. To develop SSF for keratinase production moisture content in the medium was optimized by reducing water content between 22% to 77% in

comparison with the production medium(SPM) taken earlier for submerged state fermentation which was considered as 100%. MBF11 exhibited keratinase activity of 293 KU/ml in controls. Activity of 290 KU/ml was observed even with 77% moisture content. The activity increased marginally to 300 KU/ml with 55% reduction and to 338KU/ml with 33% moisture content compared to controls. Further reduction to 22% of the original moisture content, reduced the keratinase activity drastically with only 35KU/ml of resultant activity. MBF20 exhibited a maximum activity of 338KU/ml at 33% moisture followed by activities of 295 KU/ml at 55% and 292KU/ml at 77% moisture content respectively. Least activity of 35KU/ml was observed at 22% moisture. Keratinase yield of 370 KU/ml was observed for MBF21 at 33% followed by 55 % and 77% of moisture content. MBF45 also exhibited maximum activity of 365 KU/ml at 33%. Keratinase activities of the submerged controls were 293KU/ml for MBF11, 295KU/ml for MBF20, 277KU/ml for MBF21, 246 KU/ml for MBF45 respectively. At 33%, the medium texture was semi solid. There was a significant drop in keratinase production for all the four isolates on reduction beyond 33% moisture content. Hence SPM medium with one third reduction in moisture content from 100ml to 33ml resulted in a semi solid media that was suitable to support the production of keratinase enzyme. The yield of keratinase was on an average 25% higher at the SSF conditions as compared with corresponding SmF conditions. The results of optimization are summarized in Fig1 & 2.

The keratinase production was determined at three different feather substrate concentrations between 1-4% in the optimized SSF conditions to determine optimum substrate concentration. The production of keratinase increased with increase in substrate concentrations from 1 to 2%. At 1% concentration which was also treated as control the keratinase activity was in the range of 344-381KU/ml for the test culture. The keratinase yield increased to 415 – 442KU/ml for the MBF cultures at 2% feather substrate concentration. There was no further significant increase in keratinase yield with increased substrate concentration to 3 and 4%. Hence 2% substrate concentration was considered to be optimum for keratinase production by SSF. The results are summarized in Tables 1-4.

Fig 1: Optimization of moisture content for SSF by MBF20

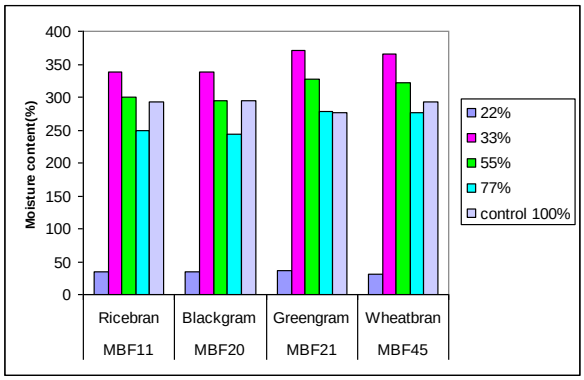

Fig 2: Optimization of moisture content of the medium for production of keratinase

Discussion

Traditionally, microbial enzymes were produced by surface cultures method using liquid or solid media. However, production of enzymes at industrial scale over the years was dominated by submerged culture fermentation method [3]. There has been a resurgence of interest in SSF for production of products using agro- industrial substrates. The numerous advantages favouring SSF over SmF include higher production yields with low capital investments as well as reasonably lower handling/ recurring costs and lack of foaming problem [4]. In general, production of keratinase in majority of reported cases required submerged conditions with the exception of keratinase reported from thermophilic anaerobic bacteria where enzyme production was favoured by static anaerobic conditions [5, 6&7]. The crucial factor in SSF system that influences the microbial growth and product yield is the initial moisture content of the substrate [8, 9&10]. As agricultural by-products were observed to support high keratinase yields in SmF, starch production media with respective bran were explored for developing SSF for keratinase production. Keratinase activity was highest with 33% moisture content of original with all isolates. Maximum resulting activity for MBF11 was 338KU/ml where as MBF20 exhibited maximum of 340KU/ml. At this moisture content the keratinase yield of MBF21 was 370KU/ml and that for MBF45 was 365KU/ml respectively. Maximum keratinase activities observed by MBF cultures in SmF controls were between 246-295KU/ml. Keratinase activities was comparable between SmF and SSF upto 66% of the moisture content of control for all the isolates. Reducing moisture content beyond 33%, of that of control (SmF), however resulted in a drastic drop in enzyme production (Fig1 & 2). The yield of keratinase was on an average higher at optimized SSF conditions as compared with corresponding SmF conditions.

Bacillus sp. have also been utilized for α-amylase production by SSF using agro-industrial residues [11].

Mitra *et al.*, 1994 [12] reviewed production of proteolytic enzymes in SSF systems. Several brans were evaluated to support the production of alkaline protease by Malathi and Chakraborthy, 1991 [13]. In their study wheat bran was found to be the best substrate for alkaline protease production in SmF systems and SSF systems. The total protease activity from 1g of bran (SSF) was equivalent to 100ml broth (SmF). Uyar and Baysal, 2004 [14] reported 30% moisture level of wheat bran to be optimum for alkaline protease production by *Bacillus* sp. whereas optimal moisture level was reported to be 74% with wheat bran for protease production by *Pseudomonas* sp. [15]. A maximum enzyme yield at substrate-to-moisture ratio of 1:2.5 has also been reported for Streptomyces sp. QG-11-3 and for *Bacillus licheniformis* [16&17]. The reduction in the yields in SSF below an optimum level of moisture is mainly attributed to decrease in porosity and/or air content of the substrate causing interference with the microbial activity by limiting oxygen transfer. Lowering the moisture level than optimum has been observed to reduce the solubility of the nutrients of the solid substrate. Increase in substrate concentration was also found to positively influence keratinase yield between 1-4% for the four *Bacillus* strains with yields increasing from 283-367KU/ml to 415-442KU/ml (Tables 1 - 4). Production of alkaline polygalacturonase by *Bacillus* sp. MG. CP-2 under various growth conditions and found maximal catalytic activities in only SSF [18, 19]. Similarly, *Bacillus subtilis* was observed to produce about 12 times more cellulase and several times more pectinase when cultivated in SSF as compared with SmF [20].

Table 1: Optimization of feather substrate for MBF11

Feather substrate	Fermentation period (Days)						
	1	2	3	4	5	6	7
	Keratinase activity (KU/ml)						
1%*	204	251	**367**	295	271	269	262
2%	126	198	250	301	373	424	230
3%	163	231	300	382	425	309	178
4%	196	222	348	415	428	317	203

* Control

Table 2: Optimization of feather substrate concentration for MBF20

Feather substrate	Fermentation period (Days)						
	1	2	3	4	5	6	7
	Keratinase activity (KU/ml)						
1%*	317	320	328	343	280	350	381
2%	275	295	246	379	442	334	391
3%	355	357	380	301	358	443	407
4%	251	278	275	328	380	443	428

* Control

Table 3: Optimization of feather substrate concentration for MBF21

Feather substrate	Fermentation period (Days)						
	1	2	3	4	5	6	7
	Keratinase activity (KU/ml)						
1%*	127	180	203	283	287	344	197
2%	183	206	290	381	415	241	108
3%	147	229	310	418	416	289	199
4%	134	204	229	358	418	253	200

* Control

Table 4: Optimization of feather substrate concentration for MBF45

Feather substrate	Fermentation period (Days)						
	1	2	3	4	5	6	7
	Keratinase activity (KU/ml)						
1%*	133	168	354	309	200	284	170
2%	103	186	328	380	430	311	154
3%	127	255	371	431	409	279	143
4%	138	280	329	381	434	205	198

* Control

Conclusions

The present study thus concludes that at the optimised condition the production of keratinase in SmF as well as SSF were comparable, the later being marginally higher. This process being potentially economical for small scale can be highly advantageous when making SSF suitable for biodegradation of feather and other keratinous substrates. Farmers/ poultry industries themselves can convert the feather into feed supplement and reuse in their own poultry waste, as the resultant fermented product could be dried and used as source of crude enzyme. This can reduce the cost of poultry farming in terms of greater yield by usage of nutritive feed supplement with value added use for waste. Thus, a novel SSF method has been proposed for the production of keratinase that can be used as an alternative and economical system.

Acknowledgements

The authors wish to thank University Grants Commission, New Delhi for the financial support and DST –CURIE Lab, SPMVV, Tiruapati for supporting the work.

References

1. Pandey A. Solid-state fermentation. Biochemical Engineering Journal. 2003; 13:81-84.
2. Jeevana Lakshmi P. 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.
3. Stanbury PF, Hall S, Whitaker. Principles of Fermentation Technology, Second Edition: Publisher: Butterworth-Heinemann, 1999.
4. Hongzhang C, Fujian X, Zhonghou T, Zuohu L. A novel industrial-level reactor with two dynamic changes of air for solid-state fermentation. Journal of Bioscience and Bioengineering. 2002; 93:211-214.
5. Friedrich AB, Antranikian G. Keratin degradation by Fervidobacterium pennavorans, a novel thermophilic anaerobic species of the order thermotogales. Applied and Environmental Microbiology. 1996; 62:2875-2882.
6. Riessen S, Antranikian G. Isolation of Thermoanaerobacter keratinophilus sp. A novel thermophilic, anaerobic bacterium with keratinolytic activity. Extremophiles. 2001; 5:399-408.
7. Nam GW, Lee Dong W, Lee Nam J, Kim B, Young C, Choe EH *et al.* Native feather degradation by Fervidobacterium islandicum AW-1, a newly isolates keratinase producing thermophilic anaerobe. Archives of Microbiology. 2002.178: 538-547.
8. Pandey A, Soccol CR, Mitchell D. New developments in solid state fermentation: I- bioprocess and products. Process Biochemistry. 2000; 35:1153-1169.

9. Ramachandran S, Patel AK, Nampoothiri KM, Francis F, Nagy V, Szakacs G, Coconut oil cake—a potential raw material for the production of α-amylase. Bioresource Technology. 2004; 93:169-174.
10. Prakasham RS, Subba Rao CH. Sarma PN. Green gram husk—an inexpensive substrate for alkaline protease production by Bacillus sp. in solid-state fermentation. Bioresource Technology. 2006; 97:1449-1454.
11. Lonsane BK, Ramesh MV. Ability of a solid state fermentation technique to significantly minimize catabolic repression of α-amylase production by Bacillus licheniformis M27. Advances in Applied Biochemistry. 1990; 35:1-56.
12. Mitra P, Chakraverty R, Chandra AL. Production of proteolytic enzymes by solid state fermentation - An overview. J Sci Ind Res. 1994; 55:439-442.
13. Malathi S, Chakraborty R. Production of Alkaline Protease by a New Aspergillus flavus Isolate under Solid-Substrate Fermentation Conditions for Use as a Depilation Agent. Applied and Environmental Microbiology. 1991; 57:712-716.
14. Uyar F, Baysal Z. Production and optimization of process parameters for alkaline protease production by a newly isolated Bacillus sp. Under solid-state fermentation. Process Biochemistry. 2004; 39:1893-1898.
15. Chakraborty R, Srinivasan M, Sarkar SK, Raghavan KV. Production of acid protease by a new Aspergillus niger by solid state fermentation. Journal of Microbiology and Biotechnology. 1995; 10:17-30.
16. Feniksova RV, Tikhomrova AS, Rakhleeva BE. Conditions for forming amylase and proteinase in surface culture of Bacillus subtilis. Microbiologica. 1960; 29:745-748.
17. Archana A, Satyanarayana T. Xylanase production by thermophilic Bacillus licheniformis A99 in solid-state fermentation**.** Enzyme Microbial Technology. 1997; 21:12-17.
18. Kapoor M, Kuhad RC. Improved polygalacturonase production from Bacillus sp. MG-cp-2 under submerged (SmF) and solid state (SSF) fermentation. Letters in Applied Microbiology. 2002; 34:317-322.
19. Bindu B, Jitender S, Kuhad R. High –level xylanase production by alkaliphilic Bacillus pumil us ASH under solid state fermentation. World Journal of Microbiology and Biotechnology. 2006; 22:1281-1287.
20. Alam M, Gomes I, Mohiuddin G, Hoq MM. Production and characterization of thermostable xylanases by Thermomyces lanuginosus and Thermoascus aurantiacus grown on lignocelluloses. Enzyme Microbial Technology. 1994; 16:298-302.