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Partial purification of keratinase from Actinomycetes screened from surrounding places of VIT University for industrial applications

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ABSTRACT

Enzymes being efficient as biocatalysts are being increasingly employed as replacements or additions for varied applications in various industries. The development of microbial keratinase can play a vital role in various other industries also. Keratinous waste in the form of feathers, which is nearly pure keratin is generated in bulk quantities in commercial poultry processing. Enzymes produced are either intracellular or extra-cellular. Extra-cellular enzymes are secreted into the medium whereas the intracellular enzymes need to be released into medium by cell disruption. In our study the recovery of Keratinase from six strains from Actinomycetes in general involves separation of biomass from the broth by filtration or by centrifugation, followed by precipitation of protein by salt or organic solvents to get a partially purified enzyme. Further steps of purification are selected depending upon the purity of the required product and its application, as recovery cost of a microbial product can vary from as low as 25% to as high as 70% of the total manufacturing costs depending on the level of purity desired for the final product The Polyacrylamide gel showed single bands in all the cases indicating homogenous nature of enzyme with one subunit. The molecular weights of the enzyme were in the range of 30 kDa for KLH104, 34-35 kDa for KLH_{99} and 43-45 kDa for KLF_3, KLF_{10}, KLF_{14} and KLF_{16} strains.

Key words: Keratinase, partial purification, SDS-PAGE, dialysis

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INTRODUCTION

Early methods for protein purification were empirical, slow and laborious. However over the years, methods are available whereby proteins can be purified to high degree of purity. There is no single procedure or set of procedures by which any and every protein can be purified but for any given protein it is usually possible to choose a sequence of separation steps that result in the desired degree of purity and a high yield. The objective in enzyme purification is to increase the purity and the biological activity of the derived protein per unit weight by getting rid of inactive or unwanted proteins while at the same time maximizing the yield of the desired fraction with minimal loss of enzyme activity. The industrial usage of enzymes thus, largely depends on their effectiveness, cost of production and safety in use. The extraction and purification steps of the fermented products are usually categorized as downstream operations. As extensive purification processes are very expensive, preparation of industrial enzymes needs to involve minimum number of purification stages that are compatible with the use. The desired purity level of the enzyme mainly depends on the purpose of its application. For pharmaceutical and other medical applications, enzymes like proteases are required with high grade of purity but in comparatively low quantities,

whereas applications in leather industry and detergent manufacture require bulk amounts of enzyme even with low purity [1,5,6]

To ensure good recovery or purification of heat labile product, speed of operations becomes an overriding factor to prevent inactivation of enzyme preparation. Sometimes, the scale of the downstream operations become a key factor in determining the commercial viability of product produced by fermentation as extraction and purification of the end product may be difficult and costly, accounting to even 70-75 percent of the total manufacturing cost. [2,6;]. Precipitation of enzymes is a useful method of concentration and is ideal as an initial step in the purification particularly on the laboratory scale. It can also be used on large scale as this process is least affected by the presence of other interfering materials compared to most of the chromatographic methods [2,3,4]. Addition of salt increases the ionic strength of the solution and causes a reduction in the repulsive effect of like charges between identical molecules of a protein. It also reduces the forces holding the solvation shell around the protein molecules. When these forces are sufficiently reduced, the protein precipitates out. Hydrophobic proteins are precipitated out at lower salt concentrations than hydrophilic proteins. Among the various salts, ammonium sulfate is convenient and effective because of its high solubility, lack of toxicity to most enzymes and its stabilizing effect on some enzymes. In addition, cost factor is also significant as this salt is cheap [1,5,7]. In large-scale processes however, this salt at high concentrations tends to form dense solution, posing problems to the collection of the precipitated enzyme by centrifugation. The salt also releases gaseous ammonia, particularly at alkaline pH. Though ammonium sulfate is the most widely used salt, some enzymes do not survive ammonium sulfate precipitation. In those cases other salts are substituted. Organic solvents are not widely used on large scale because of their higher cost, inflammability, and the tendency of proteins to undergo rapid denaturation in presence of organic solvents if the temperature is allowed to rise above 0^0C [4,7,10.11].

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MATERIALS AND METHODS

All the reagents and chemicals used were of analytical or molecular grade. Media ingredients from Hi-Media, other chemicals and reagents were from SD. Fine chemicals or SRL chemicals India Ltd and molecular weight markers from Sigma Chemicals, USA were used.

The two KLH strains and four KLF strains exhibiting higher keratinolytic activity were scaled up to a volume of 200ml and the enzyme was partially purified with ammonium sulfate precipitation method. This partially purified enzyme The partially purified enzyme sample was also fractionated on DEAE cellulose column and the samples were run on SDS PAGE to determine the molecular weight of the enzyme samples. The culture filtrate and the partially purified enzyme were tested for *in vitro* dehairing activity as well as feather degrading ability and capacity to attack other keratin substrates was also attempted.

Scaling up of Keratinase Production: Scaling up is important to get the desired product in quantities sufficient enough for purification of the product and optimize the conditions for its activity. The six strains producing significantly high keratinase were scaled up proportionately to 200 ml quantities in 2000 ml conical flasks taking production media with 1% substrate in order to produce sufficient enzyme for partial purification of keratinase. The fermentation was carried out for 12 days as described earlier. The biomass of the organism was separated by centrifugation at 5000 rpm at 4^0C. The supernatant was used as the source of extra-cellular keratinase enzyme. 200ml of the above culture filtrate in each case was used for partial purification of the enzyme by salt precipitation. [8,9,14]

Preparation of cell free extract and assay of extracellular/ intracellular keratinase activity The cell free extract was prepared for assaying intracellular keratinase enzyme activity for the test organisms by adopting the method of Suneetha 2004. The organisms were grown in presence of keratin substrate. The biomass was filtered through Whatman no.1 filter to separate residual substrate and the cells were sedimented by centrifugation and the cell mass obtained was pressed in filter paper for drying. 1gm wet weight of cell mass was mixed with 1 gm of Alumina (Neutral BDH) and kept in freezer at 0^0 C for 1 hr. The frozen mass was crushed in pre-cooled motor for 45 minutes at 4^0C. The crushed mass was then suspended in 20 ml of 0.1M Tris HCl buffer (pH 7.5) and the sample was centrifuged at 8000 rpm for 10 minutes at 4^0C. The supernatant was collected and was used as the source of intracellular keratinase enzyme. The keratinase assay for the culture filtrate and cell free extract samples was carried as per the procedure described earlier, to determine the localization of the enzyme ie. if the enzyme was extracellular, intracellular or both extracellular as well as intracellular.

Partial Purification of keratinase enzyme and determination of specific activity: The solubility of native protein is influenced by pH of the environment, the solubility being minimum at isoionic point. Proteins can be differentially precipitated with salts like ammonium sulphate, or in the presence of organic solvent at low temperatures The partial purification of keratinase enzyme from culture filtrate was done by ammonium sulfate precipitation. The culture filtrate/ cell free extract was saturated with ammonium sulfate to get 30 percent saturation and the samples were incubated at 0°C for two hours to facilitate precipitation of the protein fraction. The samples were centrifuged at 15,000 rpm for 20 minutes at 0^0C. The pellet was collected and washed twice with Tris HCl buffer (0.1M pH 7.5). The filtrate was subsequently saturated to 40%, 60% and 80% respectively and the fractions were collected. The samples were dialysed against 0.01M of the same buffer for 16hrs. and these fractions were taken as partially purified enzyme.

The total protein in the culture filtrates and partially purified enzyme fractions were estimated according to the method of Lowry *et al*., 1951 using bovine serum albumin as standard and the keratinase activity of all the samples was determined by the procedure described above. The specific activity of the keratinase enzyme was expressed as keratinase units/ mg of protein.[2,10,11,12]

Purification of keratinase on DEAE cellulose column and SDS – polyacrylamide gel electrophoresis (SDS – PAGE*):* The dialysed samples of fraction of ammonium sulphate showing the highest keratinase activity were loaded on DEAE cellulose columns equilibriated with Tris- HCl buffer to further purify the enzyme, followed by washing successively with buffer containing 0.1M, 0.25M and 0.5M concentration of NaCl to elute the sample differentially. 2.5 ml fractions were collected and the absorption at 280nm was recorded and the keratinase activity of the samples was determined. [4,13]

RESULTS AND DISCUSSION

The results of partial purification of Keratinases from the six isolates of their stability and the activity of enzymes were analyzed.

Partial Purification and specific activity: The results of the assay of keratinase in cell free extracts and culture filtrate are given in Table-. The study showed that the keratinase enzyme of the test cultures was primarily extra-cellular and was secreted into the medium. Only negligible keratinase activity was detected in cell free extracts in all the strains, indicating that the enzyme produced was totally excreted out and was not retained within the cell. The partial purification of keratinase enzyme was carried from the culture filtrates by ammonium sulfate precipitation. The results of the keratinase activity of the fractions and the corresponding specific activities are given in Table-.1 The specific activity of the culture filtrate in different cultures was between 8- 13.5KU/mg of protein. Keratinase activity was detected in all the precipitated fractions with purification folds of varying degree. The maximum keratinase yield was observed at 40-60% saturation of ammonium sulfate fraction for all strains where12 to 20 fold purification was achieved.

Table-1 Assay of Cell Free Extract/ Extracellular Extracts for Keratinase Activity

Strains	Culture filterate	Cell free extract
	Keratinase units (KU / ml)	
KLH_{99}	13.2	0.024
KLH_{104}	17.8	0.014
KLF_{3}	13.8	0.001
KLF_{10}	15.1	0.062
KLF_{14}	14.0	0.049
KLF_{16}	16.8	0.081

Partial Purification and specific activity

The study showed that the keratinase enzyme of the test cultures was primarily extra- cellular and was secreted into the medium.The partial purification of keratinase enzyme was carried from the culture filtrates by ammonium sulfate precipitation.The specific activity of the culture filtrate in different cultures was between 8- 13.5KU/mg of protein.(Table-2) Keratinase activity was detected in all the precipitated fractions with purification folds of varying degree. The maximum keratinase yield was observed at 40-60% saturation of ammonium sulfate fraction for all strains where12 to 20 fold purification was achieved.

Table-2

Partial Purification of Keratinase Enzyme with Ammonium Sulfate Precipitation

Strains	Culture Filterate			Ammonium sulfate fractions											
				0 - 30%			30 - 40%			40 - 60%			60 - 80%		
	Total Protein (mg)	Total units (KU)	Specific activity (KU/mg)	Total Protein (mg)	Total units (KU)	Specific activity (KU/mg)	Total Protein (mg)	Total units (KU)	Specific activity (KU/mg)	Total Protein (mg)	Total units (KU)	Specific activity (KU/mg)	Total Protein (mg)	Total units (KU)	Specific activity (KU/mg)
KLH_{99}	302	2420	8.01	4.51	144.1	31.95	5.04	187.6	37.22	5.13	623.7	121.57	2.90	141	48.62
KLH_{104}	264	3560	13.48	3.90	230.1	59.0	4.08	307.7	75.41	5.78	989.4	171.17	2.09	145.2	69.47
KLF_{3}	280	2780	9.92	3.10	137	44.19	3.12	205.2	65.76	4.76	897.6	188.57	1.90	241	126.17
KLF_{10}	262	2820	10.76	3.24	181.2	55.92	3.12	227.5	72.91	4.16	876.8	210.76	1.98	211.2	106.66
KLF_{14}	286	2480	8.67	3.36	196	58.33	4.05	226.5	55.92	5.58	765.7	137.22	2.60	133.7	51.42
KLF_{16}	276	3340	12.10	2.21	218.4	98.82	2.24	273.6	122.14	3.72	871.1	234.16	1.92	169.2	88.12

Table-3

STABILITY OF PARTIALLY PURIFIED KERATINASE ENZYME

Period of storage at 0^0 C	Strains					
	KLH_{99}	KLH_{104}	KLF_{3}	KLF_{10}	KLF_{14}	KLF_{16}
	Keratinase units (KU / ml)					
0 time	23.1	29.1	26.4	27.4	24.7	28.1
1 month	22.7	27.3	25.6	26.0	22.6	26.6
2 months	19.1	19.3	19.0	19.6	20.1	24.7
3 months	13.1	18.0	14.3	16.6	13.1	14.6

Period of storage at room temperature in Days	Strains					
	KLH_{99}	KLH_{104}	KLF_{3}	KLF_{10}	KLF_{14}	KLF_{16}
	Keratinase units (KU / ml)					
0	23.1	29.1	26.4	27.4	24.7	28.1
1	18.9	20.1	16.9	17.9	16.8	21.7
2	14.8	12.6	12.5	13.7	15.4	17.5
3	10.4	10.3	11.0	12.0	10.8	12.6
4	9.4	9.5	8.7	7.8	6.8	7.5
5	7.4	6.4	5.5	4.9	4.6	3.7
6	6.7	5.7	4.8	3.9	4.3	2.9
7	3.2	4.1	3.6	2.3	2.9	1.6

Stability of enzyme : The stability of the partially purified enzyme was studied at 0ºC and 37ºC for various intervals of time and the results are presented in Table- 3.The keratinase enzymes was found to be quite stable when stored at

0ºC upto 2months after which there was a 45 -60% reduction in enzyme activity. When stored at room temperature the half life of the enzyme was 2-3days and the enzyme activity was reduced significantly by 7 days.

▪ Purification of keratinase on DEAE cellulose column and SDS – polyacrylamide gel electrophoresis (SDS – PAGE)

The Polyacrylamide gel showed single bands in all the cases indicating homogenous nature of enzyme with one subunit. The molecular weights of the enzyme were in the range of 30 kDa for KLH104, 34-35 kDa for KLH_{99} and 43-45 kDa for KLF_3, KLF_{10}, KLF_{14} and KLF_{16} strains.

CONCLUSION

The keratinase enzyme produced was inducible, extracellular and was efficiently excreted into the medium with negligible keratinase activity in cell free extracts. . The process being highly economical, it is estimated that adopting keratinase technology to process feathers can create 400 million dollars market based on the value of digestible protein in US alone and similar high projections worldwide are also forecasted. Thus Keratinases can play a vital role in utilization of this important and hitherto untapped source of protein waste as food and feed supplement. Further studies on the application potential of the keratinase from the native or improved strains in the condition employed in the leather industry can boost the possibility of development of indigenous keratinase technology.

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