RESEARCH ARTICLE

Investigation of environmental parameters affecting feather degradation and keratinase production by *Stenotrophomonas maltophilia* K279a

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ABSTRACT

Feathers represent a sizable portion of waste generated by poultry processing industries. This waste can be utilized for production of feather hydrolysate which can be used as animal feed. The present study investigated effect of various environmental parameters on chicken feather degradation and keratinase production by keratinolytic isolate *Stenotrophomonas maltophilia* K279a. *Stenotrophomonas maltophilia* can utilize feathers as a sole source of carbon, nitrogen and energy. Feather degradation and keratinase activity gradually increased and reached an optimum level on the 6th day. Maximum feather degradation, keratinase activity and bacterial growth were observed under shaker condition. The bacterium was able to grow at pH 5.0 to 9.0 with optimum feather degradation (73.34 %) and keratinolytic activity (44.30 units/ml) observed at pH 7.5. Optimum feather degradation (75.6 %) and keratinolytic activity (45.62 units/ml) was observed at 37 ºC. 2 % Inoculum volume and 1% feather concentration was found to be optimum for feather degradation and keratinase production. Amongst various co-carbon sources investigated, feather degradation and keratinase activity increased in presence of 1 % glucose, glycerol, maltose and lactose. Similarly, amongst co-nitrogen sources; 0.1 % concentration of peptone, tryptone, yeast extract and ammonium sulphate had positive effect on feather degradation and keratinase production. Presence of reducing agent, sodium sulphite (0.1 %) in growth media caused significant increase in feather degradation. Feather degradation and keratinase production were not affected by the presence of surface tension reducers in growth media. Results of this study can help in designing media with appropriate nutrients and incubation conditions, which can lead to increased feather degradation under submerged condition.

Keywords: Keratinolytic activity, *Stenotrophomonas maltophilia*, feather degradation.

INTRODUCTION

Amongst various agriculture segments in India, poultry is considered to be one of the fastest growing segments, growing at a rate of 8 % to 10 %. Thus in the coming years, there will be significant increase in generation of poultry waste which, if not handled properly, can lead to environmental pollution and health hazard. One of the major constitutions of poultry waste is feathers. Feathers are almost pure keratin protein and hence can be used as a cheap alternative for production of protein rich animal feed. Current methods to convert feathers into animal feed include physical and chemical processing. However, these processes require significant amount of energy. Further, these processes also cause destruction of certain essential amino acids, reducing nutritional value of the feed. Chemicals used in feather processing are responsible for environmental pollution (Jin-Ha Jeong, 2010). Biodegradation of feathers by keratinolytic microorganisms is seen as a potential alternative to reduce energy consumption, improve nutritional value and reduce environmental pollution.

Unlike most proteins which are easily degraded by common proteolytic enzymes like papain, pepsin and trypsin, feather keratin protein is not degraded by these enzymes. Feather protein keratin is stabilized by disulphide bonds, hydrogen bonds and hydrophobic interactions. Keratinolytic proteases produced by certain microorganisms play an important role in feather degradation in nature. A variety of bacteria, actinomycetes and fungi are known to produce keratinolytic enzymes. Amongst bacteria, keratinolytic activity has been widely documented in Gram-positive bacteria *Bacillus* spp. and *Streptomyces* spp. Amongst Gram-negative bacteria, keratinolytic activity has been reported in *Xanthomonas maltophilia*, *Vibrio* sp., *Stenotrophomonas* sp. D-1, and *Chryseobacterium* sp. strain kr6 (Gupta and Ramnani 2006). These microorganisms can be exploited for biodegrading of keratinaceous waste, which can be converted into valuable product such as animal feed, nitrogen rich organic fertilizer, etc. Keratinase enzyme produced by these organisms is a valuable resource for food industry, detergent producing industry, leather industry, cosmetic industry, etc (Saber et al., 2010). Thus, it is important and necessary to determine conditions which can lead to increased feather degradation and improved enzyme production for such applications.

Recently, we have isolated and characterized a novel feather degrading Gram-negative bacterium, *Stenotrophomonas maltophilia* K279a. The organism can grow by utilizing feather as the sole source of carbon, nitrogen and energy. The aim of the present study was to investigate the effect of various environmental parameters on chicken feather degradation and keratinase production by keratinolytic isolate *Stenotrophomonas maltophilia* K279a.

MATERIALS AND METHODS

Microorganism and growth medium: *Stenotrophomonas maltophilia* K279a used in this study was isolated from a poultry waste dumping site in Mumbai. Feathers were procured from a local poultry shop. They were washed thoroughly with tap water followed by final washing with distilled water. The feathers were dried at 40 ºC and cut into pieces of 1 – 2 cms. Feather basal media (FBM) used for growing the isolate had the following composition: NaCl, 0.5 gm; K_2HPO_4, 0.3 gm; KH_2PO_4, 0.4 gm; Na_2SO_3, 0.5 gm and Feathers, 10 gm; pH 7.5; D/W, 1000 ml. The medium was sterilized by autoclaving.

Feather degradation (FD):

Feather degrading potential of the organism was tested by inoculating 1 ml of freshly prepared suspension (approximately 10^8 cells/ml) in 100 ml of sterile FBM broth in 250 ml Erlenmeyer flask. The medium contained whole feathers as a sole source of carbon, nitrogen and energy. The incubation was carried out at 37ºC for 6 days. Percentage feather degradation was estimated by gravimetric method (Suntornsuk and Suntornsuk, 2003).

Keratinase Activity (KA):

Keratinase activity was determined by the Sigma Aldrich method with minor modification (Shah and Vaidya, 2017).

Effect of static and shaker conditions on FD:

For this, two sterile FBM flasks were inoculated with *Stenotrophomonas maltophilia* K279a. One flask was incubated in shaker condition (90 rpm) and other flask at static condition. The incubation was carried out at 37ºC for 6 days. % FD and KA was estimated at the end of 6th day by the method described above. Increase in cell number was measured by pour plate technique and reported as CFU/ml.

Effect of rotation speed on FD:

The effect of rotation speed (rpm) on FD and keratinase production was studied by incubating the inoculated FBM broth at different rpm (90, 120, 150 & 180) at 37 ºC for 6 days. % FD and KA was estimated at the end of 6th day by the method described above.

Time course of FD:

The effect of incubation period on FD and keratinase production was studied by incubating the inoculated FBM broth at 90 rpm and 37 ºC for 3, 4, 5, 6 and 7 days. Un-inoculated FBM medium was kept as control. % FD, KA and increase in cell number was estimated by the method described above.

Effect of initial medium pH on FD:

The test isolate was inoculated in 100 ml of sterile FBM flasks having pH values of 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0 and incubated at 90 rpm and 37 ºC for 6 days. Un-inoculated FBM flasks having the same pH values were kept as controls. After incubation, % FD and KA were estimated by the method described above.

Effect of incubation temperature on FD:

FBM flasks having pH 7.5 were inoculated with the test isolate. The flasks were incubated on incubator-shaker maintained at following temperatures viz. 27˚C, 32˚C, 37˚C and 42˚C for 6 days. Un-inoculated FBM flasks maintained at the same temperatures were kept as controls. % FD and KA were estimated by the method described above.

Effect of initial feather concentration on FD:

Sterile FBM flasks with 0.5%, 1%, 1.5% and 2% feathers were inoculated with the test isolate. Un-inoculated FBM flasks having same concentration of feathers were used as controls. The flasks were incubated on incubator-shaker (90 rpm) at 37 ºC for 6 days. % FD and KA were estimated by the method described above.

Effect of inoculum volume on FD:

Sterile FBM with 1% feathers was inoculated with 1 %, 2 % and 3% of freshly prepared suspension (approximately 10^8 cells/ml) of the test isolate. Un-inoculated FBM was kept as control. The flask was incubated on incubator-shaker (90 rpm) at 37 ºC for 6 days. % FD and KA was estimated by the method described above.

Effect of various co-carbon and co-nitrogen sources on FD:

Sterile FBM was supplemented with 1% co-carbon sources such as Dextrose, Sucrose, Fructose, Galactose, Lactose, Maltose, Mannitol, Glycerol, Xylose and Starch and 0.1% co-nitrogen sources such as Peptone, Yeast Extract, Tryptone, Meat Extract, Beef Extract, Ammonium Sulphate, Sodium Nitrate, Ammonium Chloride and Ammonium Nitrate. The flask was inoculated with the test isolate. Un-inoculated FBM was kept as control. The flask was incubated on incubator-shaker (90 rpm) at 37 ºC for 6 days. % FD and KA was estimated by the method described above.

Effect of reducing agents and detergents on FD:

Sterile FBM was supplemented with 0.1% reducing agents such as Sodium Sulphite, Di-thiothreitol and β-mercaptoethanol and 0.1 % detergents such as Tween 80, SDS and Triton X 100. The flasks were inoculated with the test isolate. Un-inoculated FBM flasks were kept as controls. The flasks were incubated on incubator-shaker (90 rpm) at 37 ºC for 6 days. % FD and KA was estimated by the method described above.

Statistical analysis:

All the experiments were carried out in triplicates. The results presented are mean ± standard deviation.

RESULTS AND DISCUSSION

Stenotrophomonas maltophilia K279a is able to grow on feather basal medium containing feathers as a sole source of carbon, nitrogen and energy (figure 1). The organism, when grown in FBM under submerged condition at 37 ºC for 6 days, caused significant degradation of feathers (76.61%). Keratinase activity in the culture free supernatants was 47.91 Units/ml. Media pH gradually increased from 7.5 at Day 1 to 8.5 at Day 6. Alkalinization of culture media is one of the characteristic features of keratin degradation and is due to release of alkaline products from breakdown of keratin into peptides and amino acids, which further undergo deamination to release amines and ammonia (Nereida et. al., 2009). Kaul and Sumbali (1997) proposed that fungi having strong keratinolytic ability rendered the culture medium more alkaline than those that were less keratinolytic.

Maximum feather degradation, keratinase activity and bacterial growth were observed under shaker condition as compared to static condition (Figure 2).

Figure 1: A Residual feathers after treatment with *Stenotrophomonas maltophilia* K279a; B – Standard Proteinase K graph using Keratin Azure k 8500 as a substrate.

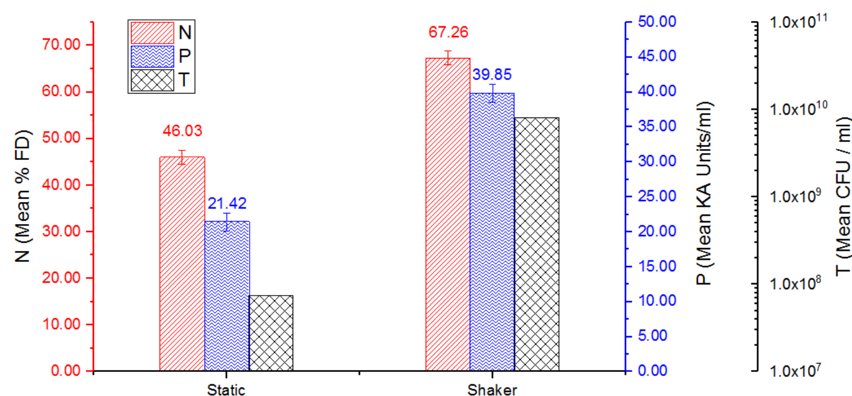

Figure 2: Effect of static and shaker conditions on cell number, FD and KA.

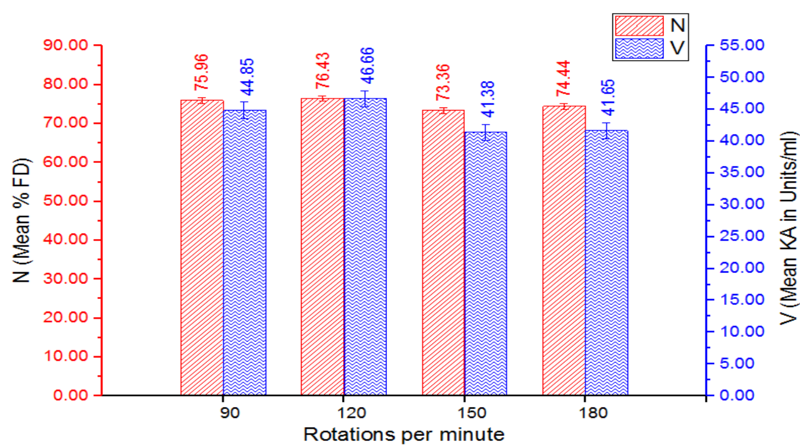

Figure 3: Effect of rotation speed on FD and KA.

The rotation speed of 120 rpm resulted in maximum feather degradation and keratinase production. However, no significant difference between feather degradation and keratinase activity was observed at 90 rpm and 120 rpm. There was negligible reduction in feather degradation and keratinase production at 150 and 180 rpm (Figure 3). In *Bacillus* sp. FK 46, maximum increase in cell number, feather degradation and keratinase production were reported at 120 rpm as compared to lower rpm (Suntornsuk and Suntornsuk, 2003).

The time course of feather degradation and keratinolytic activity by *Stenotrophomonas maltophilia* K279a culture grown in a feather basal medium is shown in Figure 4. The maximum keratinolytic activity was about 45.83 Units/ml after 6 days of cultivation and decreased to 41.44 Units/ml on 7th day. Similarly, maximum feather degradation of around 77 % was observed on 6th day and remained constant on 7th day.

The time course of keratinase production and feather degradation followed similar pattern. Feather degradation was accompanied by change in media pH

from 7.5 to 8.3 at the end of 6th day, indicating proteolysis and release of alkaline products in the medium. Cell number (Cfu/ml) gradually increased to maximum on 5th day (9.51 X 10^9) and decreased thereafter. Geun-Tae Parka and Hong-Joo Son (2009) reported complete degradation of chicken feathers by *B. megaterium* F7-1 after 7 days of incubation at 30 ºC. Similarly, Williams *et al.* (1990) demonstrated that *Bacillus licheniformis* PWD-1 degraded chicken feather completely at 50 ºC in10 days.

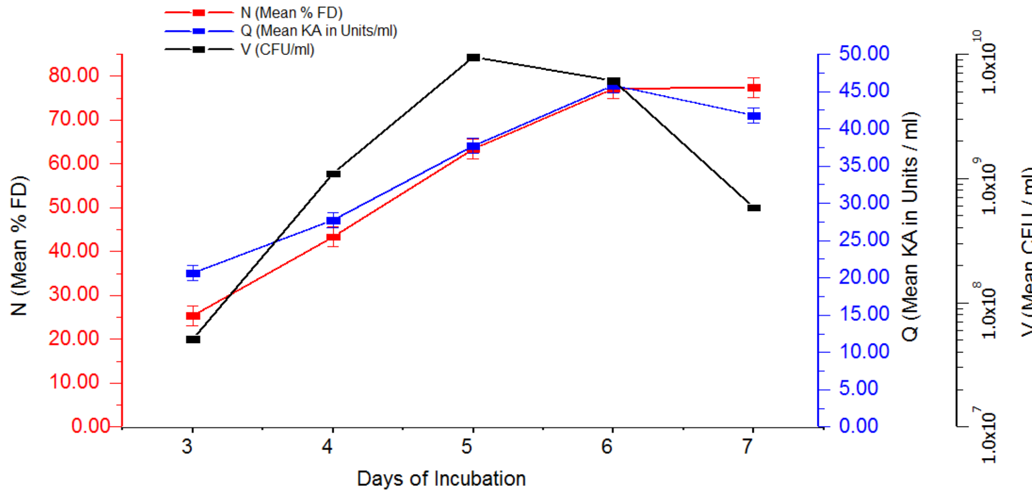

Figure 4: Time course of change in cell number, feather degradation and keratinolytic enzyme production by *Stenotrophomonas maltophilia* K279a.

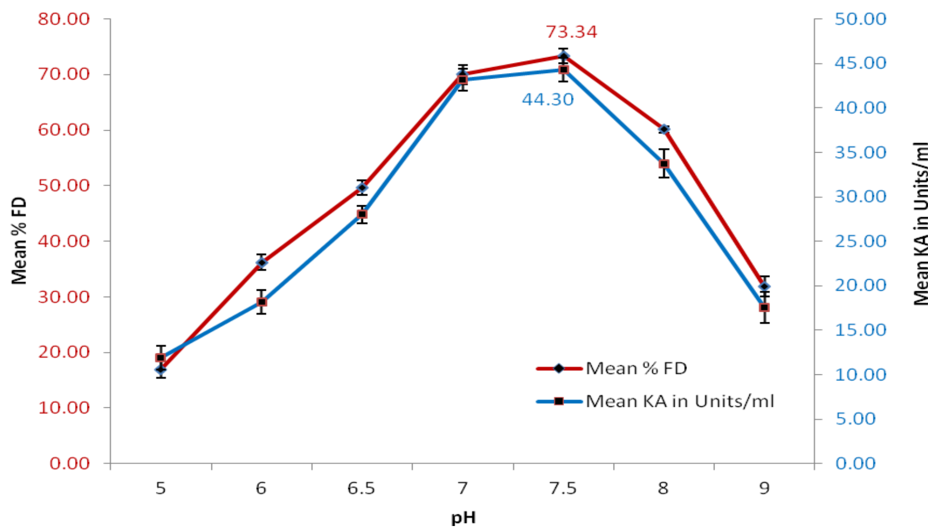

Figure 5: Effect of initial media pH on feather degradation and keratinolytic enzyme production by *Stenotrophomonas maltophilia* K279a.

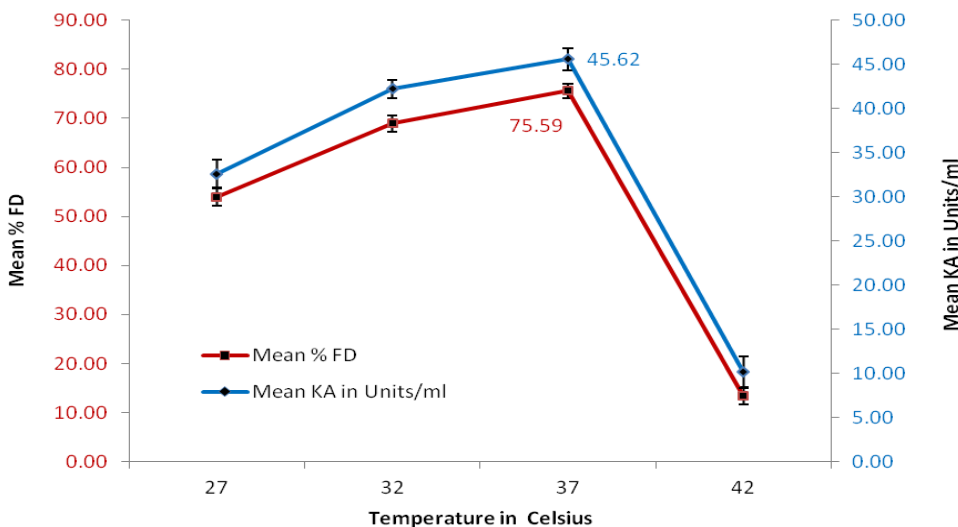

Figure 6: Effect of incubation temperature on feather degradation and keratinolytic enzyme production by *Stenotrophomonas maltophilia* K279a.

The optimum pH for feather degradation and keratinolytic enzyme production was determined by growing *Stenotrophomonas maltophilia* K279a at pH 5.0 – 9.0 and 37 ºC for 6 days. As shown in Figure 5, maximum feather degradation (73.34 %) and keratinolytic activity (44.30 Units/ml) were observed at pH 7.5. The enzyme activity was considerably lower in the pH range of 5.0 – 6.5, while it was higher in pH range 7.0 to 8.0. The organism was able to grow over a wide range of pH 5.0 – 9.0. In *B. licheniformis* keratinase production was optimum in neutral conditions i.e. in pH range of 7.0 –7.5, and for *B. subtilis* it was best in an alkaline condition (pH 8.0–8.5) (Wang and Shih, 1999). *Stenotrophomonas maltophilia* DHHJ exhibited maximum production of keratinase at pH 7.5 (Zhang et. al., 2009). Keratinolytic enzyme production in *Stenotrophomonas maltophilia* R13 was observed in pH range of 4.0–11.0, with an optimum production at pH 7.0. (Jin-Ha Jeonga et. al., 2010).

The effect of incubation temperature on Feather degradation and keratinase production is shown in figure 6. Maximum feather degradation of 75.6 % and keratinase activity of 45.62 units / ml were observed at 37 ºC after 6 days of incubation in shaker condition. Brigitte Bockle *et al.* (1995) reported that *Streptomyces pactum* DSM40530 partially degraded native chicken feathers at 50 ºC. For *Stenotrophomonas maltophilia* DHHJ, maximum keratinase production was reported at 40 ºC. Similarly, in *Stenotrophomonas maltophilia* R13, optimum temperature for the enzyme production was 30 ºC. Keratinase production in *Chryseobacterium* strain kr6 and *Vibrio* strain kr2 and was optimum at 25 ºC and 30 ºC respectively (Sangali and Brandelli, 2000; Riffel *et al.*, 2003).

S. maltophilia was grown in the medium containing 0.5 % to 2 % feather concentrations. The maximal feather degradation was observed at 1 % feather concentration. However, 1 % and 1.5 % feather concentration showed maximum keratinase production (Figure 7). Further, inoculum volume of 2 % (v/v) resulted in maximum feather degradation and keratinase enzyme production (Figure 8). For, *Bacillus subtilis* KD-N2, inoculum volume of 2 % was found to be optimum for keratinase production (Cai C., and Zheng X., 2009).

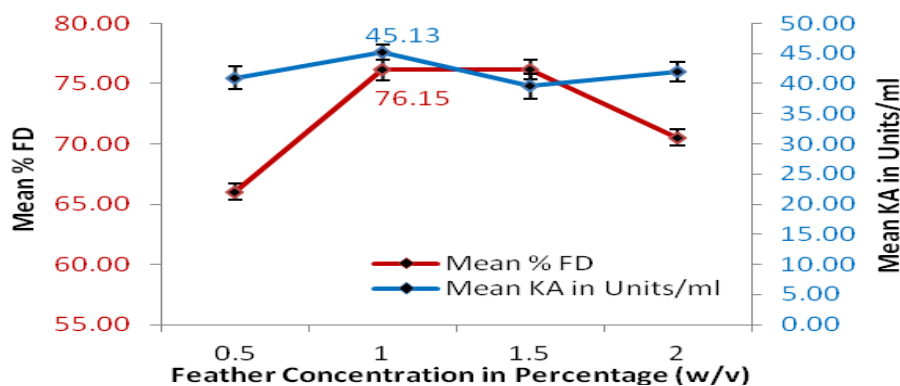

Figure 7: Effect of feather concentration on feather degradation and keratinolytic enzyme production by *Stenotrophomonas maltophilia* K279a.

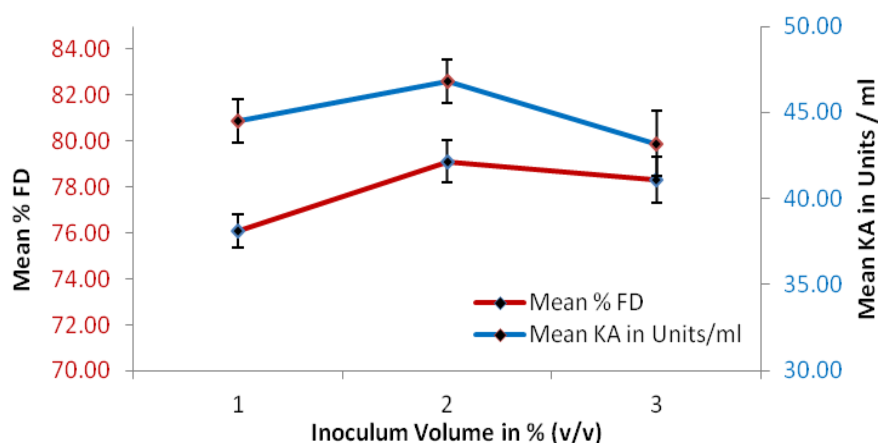

Figure 8: Effect of inoculum volume on feather degradation and keratinolytic enzyme production by *Stenotrophomonas maltophilia* K279a.

Table 1: Effect of various carbon, nitrogen, reducing agent and surface tension reducers on feather degradation and keratinolytic enzyme production by *Stenotrophomonas maltophilia* K279a.

	% FD	% increase or decrease in FD	KA (Units/ml)	% increase or decrease in KA
Co-carbon (1 %)				
Basal FBM	76.61 ± 1.92	0.00	47.91 ± 1.58	0.00
Fructose	74.85 ± 1.50	-2.29	41.93 ± 1.26	- 12.48
Galactose	73.67 ± 1.71	-3.84	40.40 ± 1.08	- 15.67
Glucose	85.13 ± 1.15	11.12	58.76 ± 1.99	22.65
Glycerol	79.11 ± 1.12	3.26	54.66 ± 1.67	14.09
Lactose	78.87 ± 1.21	2.95	49.30 ± 1.94	2.91
Maltose	78.90 ± 1.22	2.99	50.97 ± 1.26	6.39
Mannitol	74.53 ± 0.79	-2.71	42.91 ± 2.66	- 10.44
Starch	73.97 ± 0.99	-3.45	41.83 ± 1.99	- 12.69
Sucrose	73.36 ± 2.26	-4.25	43.18 ± 1.07	-9.86
Xylose	71.76 ± 1.83	-6.33	41.79 ± 1.19	- 12.77
Co-Nitrogen (0.1 %)				
Basal FBM	75.72 ± 1.52	0.00	46.80 ± 2.40	0.00
Meat Extract	72.43 ± 0.66	-4.34	41.38 ± 0.32	- 11.59
Peptone	79.66 ± 0.83	5.21	54.45 ± 1.62	16.35
Tryptone	78.85 ± 0.70	4.14	51.81 ± 1.89	10.70
Yeast Extract	78.65 ± 0.77	3.87	52.99 ± 1.15	13.22
Ammonium Chloride	72.33 ± 1.42	-4.47	42.07 ± 1.85	- 10.10
Ammonium phosphate	74.14 ± 0.77	-2.08	44.09 ± 1.27	- 5.79
Ammonium Sulphate	78.48 ± 0.62	3.65	52.71 ± 0.55	12.63
Sodium Nitrate	76.36 ± 1.07	0.85	48.96 ± 1.67	4.61
Reducing agents (0.1 %)				
Basal FBM	74.12 ± 0.91	0.00	44.16 ± 0.75	0.00
β-mercaptoethanol	72.47 ± 0.54	- 2.23	42.00 ± 1.69	- 4.89
Di-thiothretol	73.31 ± 1.16	- 1.09	45.13 ± 1.82	2.20
Sodium Sulphite	86.96 ± 0.27	17.32	60.71 ± 1.48	37.48
Detergents (0.1%)				
SDS	73.21 ± 0.77	- 1.23	44.71 ± 1.48	1.26
Tween 80	70.05 ± 0.67	- 5.49	40.40 ± 1.37	- 8.51
Triton X 100	73.21 ± 1.39	- 1.23	42.70 ± 1.66	- 3.31

The influence of the addition of various co-carbon and co-nitrogen sources, reducing agents and surface tension reducers to the feather basal salt medium is shown in table 1. Addition of co-carbon sources such as glucose and glycerol resulted in significant increase in keratinase production, whereas presence of maltose

resulted in slight increase in enzyme production. In case of *B. megaterium* F7-1, presence of fructose, galactose, and glucose in growth medium resulted in slight increase in keratinase enzyme production (Geun-Tae and Hong-Joo, 2009). In *S. maltophilia* R13, increased keratinase activity was observed for various co-carbon sources, of which glucose promoted the greatest degree of keratinolytic enzyme production and cell growth (Jin-Ha Jeong et al., 2010). Conversely, In *Stenotrophomonas sp*. D-1 keratinase production is partially inhibited by glucose (Yamamura et. al., 2002). Similarly, in *Streptomyces* MS2, glucose and starch showed negative effect on keratinase production (Mabrouk, 2008). Amongst co-nitrogen sources, peptone, tryptone, yeast extract and ammonium sulphate caused significant increase in keratinase production. In *Stenotrophomonas sp*. D-1 keratinase production increased in presence of yeast extract. Similarly, keratinolytic enzyme production by *S. maltophilia* R13 increased in presence of polypeptone and decreased in presence of yeast extract (Jin-Ha Jeong et al., 2010). In *B. megaterium* F7-1 keratinolytic enzyme production is positively influenced by tryptone and yeast extract. Usually, the effects of co-carbon and co-nitrogen sources on keratinase production vary according to the species and carbon or nitrogen source and their concentration (Cai and Zheng, 2009). Thus, it is necessary to optimize composition of culture media on a case-by-case basis to improve keratinase production. Increased enzyme production in presence of some co-carbon and co-nitrogen sources can be due to increased cell growth. Presence of sodium sulphite in the culture medium caused significant increase in feather degradation and keratinase production. Feathers are highly stable to degradation due to large number of di-sulphide bonds in keratin protein. Presence of sodium sulphite in culture medium may be responsible for reduction of di-sulphide bonds in keratin protein resulting in increased feather degradation. Presence of surface tension reducers in medium did not cause any significant difference in feather degradation and keratinase production.

CONCLUSION

In this work, we have investigated various environmental parameters that affect feather degradation and keratinase production by keratinolytic isolate *Stenotrophomonas maltophilia* K279a. *Stenotrophomonas maltophilia* shows maximum feather degradation and keratinase production in shaker condition at 37 ºC and pH 7.5. Addition of glucose, glycerol, maltose, peptone, tryptone, yeast extract, ammonium sulphate and sodium sulphite in the growth medium had a positive effect on feather degradation and keratinase production. The results of this study can help in determining incubation conditions and designing appropriate fermentation medium with feathers and for keratinase and feather hydrolysate production.

Conflicts of interest: The authors stated that no conflicts of interest.

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