Isolation of Keratinolytic Bacterial Strains from poultry farm Soil Samples; Production, Optimization and Thermal Characterization of the Keratinase Enzyme(s)

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Summary: Millions of tons of chicken feather waste are generated annually worldwide. In Pakistan, Poultry industries are ranked among the most important sectors contributing economically owing to the enormous consumption of chicken meat; on the other hand, it causes the accumulation of huge quantities of keratinous waste because of poor solid waste management strategies. Keratin is a major constituent in the structure of feathers, mainly composed of beta keratin. Being enriched in cysteine residues, keratin offers resistance towards most of the proteases. However, these proteins can be attacked by microbial keratinases. A total of 54 bacterial strains isolated from poultry waste (feathers) samples were screened for extracellular enzyme production such as amylase, gelatinase, caseinase and keratinase. On the basis of complete feather degradation profile in minimal basal medium within 72 hrs, studies on keratinase enzyme production were conducted with bacterial strains named as MW45 and MW48. Keratinase production from both the producer strains was found optimum at 45°C and maximum keratinase production was noticed at pH 9. Upon thermal characterization, the keratinase enzyme was found optimally active at 60°C and 70°C by MW45 strain and MW48 strain respectively.

Keywords: Chicken Feather waste, Keratinase, Bioremediation, Optimization, Thermal characterization, Solid waste management.

Introduction

Feather waste is mostly a keratinous waste generated largely from poultry industries. Globally, about 2 million tons feather waste is generated from chicken feathers on annual basis [1]. According to the poultry meat production data 2014, 776,000 tons chicken meat was produced in Pakistan in 2012, and it has increased up to 1.6 percent in 2014. Feathers constitute about 5-7% of a chicken's body weight [2]. It can be estimated that thousands of tons of feather waste is generated annually in Pakistan. Feathers are highly enriched in beta keratin i.e. up to 90%. These keratins are insoluble, mechanically stable, and fibrous structural proteins; having beta turns, and are linked through disulphide bridges by means of cysteine residues. Apart from feather waste, millions of tons of other keratinous waste is generated from live-stock population [3]. Keratin is a main constituent of skin, and its appendages like nails, hair, wool, feathers, hoofs, horns, beaks, scales of animal, and other epithelial coverings [4, 5].

As keratin is a recalcitrant protein that resists degradation when treated with different proteases such as trypsin, pepsin and papain, there is an ultimate need to search for such proteases that can actively act upon on this huge keratinous waste [6]. Fortunately, a number of microorganisms can potentially act on this mechanically stable protein by means of producing extracellular enzymes, known as **keratinases**. A number of genera from bacteria, fungi, and actinomycetes have been documented for keratinase production such as *Bacillus, Kocuria, Lysobacter, Microbacterium, Nesternokia, Proteus, Pseudomonas, Stenotrophomonas, Xanthomonas, Chryseobacterium* [7, 8].

Cost effective production of keratinases owing to the bulk availability of feather waste makes it a potential candidate for industrial applications. Currently, these keratinolytic enzymes are found more efficient for deep stains removal [9]. Intact hair removal from leather can be done by detaching hair root follicle keratin using keratinases without any adverse effect. Keratinases can also be used as a replacement of heat treatment providing improved digestibility and nutritional content of feed [10].

Considering the environmental concerns and broad spectrum applications of keratinase enzyme, isolation of keratinolytic bacterial strains and production optimization was attempted in the current study.

Experimental

Isolation of the potential bacterial strains

A total of four samples were collected from a local poultry farm in the pre-autoclaved screw capped bottles. Serial dilutions of the samples were

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prepared till 10^{-8} and were spread onto nutrient agar plates using spread plate technique. The colonies that appeared were purified on nutrient agar plates. The purity of isolates was further checked by Gram's staining. These cultures were preserved by means of glycerol stocks [11].

Temperature- tolerance profile

Thermo-tolerance of the isolates was checked by incubating the inoculated plates for 24 h at 4°C, and different temperatures ranging from 25-75°C with stride of 5°C [12].

Enzyme based hydrolytic Profiles of the isolated strains

Zymogenic profiles of the isolates were analyzed using plate screening assay with respective substrates [13]. Enzyme index for each enzyme was calculated as follows:

$$\text{Enzyme index} = \frac{\text{Zone of hydrolysis (mm)}}{\text{Colony diameter (mm)}}$$

Amylase activity

For amylase activity, 1% (w/v) starch in LB medium (glucose 0.1%, yeast extract 0.25%, and agar 1.8%) was used. The isolates were spot inoculated and incubated at 37°C for 24 h. Zone of hydrolysis was measured after exposing the plates to iodine crystals for 5 min [14].

Gelatinase activity

The isolates were screened for gelatinase activity by using protease specific medium containing K_2HPO_4 0.2%, glucose 0.1%, peptone 0.5%, gelatin 1.5%, and agar 1.5% at 37°C. The diameter of clear zone was measured after 24 h of incubation [15].

Caseinase activity

To analyze caseinase activity, all isolates were spot inoculated onto the medium containing 0.1% glucose, 0.25% yeast extract, 1% casein, 0.5% NaCl, 1.8%agar. Plates were inoculated at 37°C for 24h. Zone of hydrolysis was measured in mm, and enzyme index was calculated [15].

Keratinase screening

The isolates were screened for keratinase production on the medium containing 0.5% keratin azure, 0.03% K_2HPO_4, 0.04% KH_2PO_4, 0.05% NaCl, 0.01% $MgCl_2$ and 2% agar [16]. Plates were incubated at 37°C for 48 h. A light blue zone of precipitation indicated the keratin degradation.

Evaluation of keratinolytic strains for complete feather degradation

Complete feather degradation by these isolates was determined by using 20ml minimal medium (0.03% K_2HPO_4, 0.04% KH_2PO_4, 0.05% NaCl, 0.01% $MgCl_2$) with 0.5% (w/v) native feathers as the sole source of carbon. 1ml of the freshly grown (24 h old) cultures was used and kept at 33°C at 100rpm. Feather degradation was monitored every after 24 h.

Spectrophotometric analysis of keratinase Production

After complete feather degradation, culture broths of each keratinolytic strains were centrifuged and cell free extract was quantitively assessed for keratinase production by running a standard enzyme assay for proteases.

Enzyme assay

130µl of casein solution (0.66% w/v) + 25µl of crude enzyme extract

↓

Incubation at 37°C for 20 minutes

↓

Addition of 130µl of 110mM TCA

↓

Incubation at 37°C for 20 minutes

↓

Centrifuged at 2500 rpm for 10 minutes

↓

250µl of the supernatant transferred to the separate tube

↓

Addition of 625µl of 500mM sodium carbonate solution + 125µl of Folin and Ciocalteu's Phenol Reagent

↓

Incubation at 37°C for 30 minutes

↓

Optical density recorded at 660nm by using spectrophotometer

One unit will hydrolyze casein to produce a color equivalent to 1.0µmole (181µg) of tyrosine per minute at pH7.5 at 37°C (color by Folin and Ciocalteau's Reagent).

$$\text{Unit/ml enzyme} = \frac{\mu\text{mol of Tyr} \times \text{volume}}{\text{Sample vol.} \times \text{Reaction time} \times \text{Vol. assayed}}$$

Optimization of keratinase production

Optimization of the physical parameters i.e. temperature and pH was followed by selected potential strains (MW45 & MW48), employing One Variable at A Time (OVAT) approach [17].

Effect of Incubation Time

The optimum incubation time for maximum keratinase production was assessed by keeping the culture flask in shaking incubator for about 72 h at 40°C (150rpm). Enzyme activity was then quantified every after 24 h using standard assay protocol.

Temperature optimization

The seed culture of the selected strains was prepared, freshly grown (24hrs old) cultures was then inoculated in 20ml of minimal medium with 0.5% (w/v) of native feathers. These mediums were incubated at different temperatures ranging from 40°C to 55°C with stride of 5°C at 150rpmfor 72 hrs [18].

Effect of substrate

The keratinolytic potential of the isolates was evaluated on both the soluble keratin and the native feathers (0.5%w/v). Enzyme production was evaluated by performing enzyme assay after 72 hrs at 45°C.

pH optimization

Effect of pH on keratinase production was observed at different pH values i.e. from 4 to 11 with increment of 1 unit. Purposely, pH of the minimal media broth (0.5% native feathers as substrate) was adjusted prior inoculation using 1M NaOH and 1M HCl. After inoculation (5% v/v), these flasks were incubated at 45°C for 72 hrs with vigorous shaking (150rpm). Thereafter, Enzyme assay was run as per standard protocol.

Thermal Characterization of Keratinase

Effect of temperature on enzyme activity

Temperature dependency of the keratinase activity was analyzed by incubating the enzyme-substrate reaction mixture at different temperatures ranging from 30°Cto 100°C with stride of 10°C for 20 minutes. Enzyme activity was measured at 660nm after running the complete assay procedure.

Thermo-stability evaluation of the crude enzyme extracts

Thermal stability of the keratinase enzyme was determined by incubating the enzymatically active fractions of cell free supernatant at different temperatures i.e. from 30°C to 100°C with stride of 10°C for 1 hour. Thereafter, enzyme assay was performed as per standard assay protocol.

Statistical analysis

All the experiments were performed in triplicates and results were expressed as mean±S.D.

Results and Discussion

Feather waste generated from poultry industries is largely keratinous in nature [19]. In Pakistan, poultry industries are one of the economically important sectors due to the enormous consumption of chicken meat [20]. On the other hand, disposal of feather waste is a great environmental issue, leading to the pollution of both land and underground water resources. As keratin is a defiant protein**,** its degradation demands such proteases that can actively recycle the feather waste [21]. Thus, present study focused on the production of keratinase enzyme from the indigenous bacterial strains.

A total of 54 bacterial strains were isolated from the soil samples collected from a poultry farm as the farm's soil is rich in feather waste comprised of ≥ 90% keratin. The same approach has been followed in a number of study reports for the isolation of keratin degrading microorganisms [22].Majority of the strains were found as Gram positive. Microscopic examination revealed 20 strains as cocci, 30 as rods, and 01 as cocco-bacilli shaped. Three of these strains were found to be Gram negative, short rods. This may be explained by means of Winogradsky's classification which states that being spore formers, Gram positive bacteria resist fluctuating environmental conditions such as high

temperature, desiccation, variable pH and exposure to various chemicals and radiations.

The growth profile of the isolates was recorded at different temperatures such as at 4°C, and from 25°C to 75°C. It was observed that all the isolates were able to grow at a temperature range of 25-45°C. Most (72%) of the strains were able to grow up to 55°C whereas, 55% of the isolates showed growth up to 75°C (fig. 1). It is reported that keratinolytic microorganisms can grow up to varied range of temperature i.e. 25°C to 55°C, and pH range of 6.0-9.0 [10]. Lakshmi *et al.,* (2013) also found that the keratinolytic strains BF11 and BF21 (isolated from local poultry farm) were able to grow within a range of 27°C to 55°C [23].

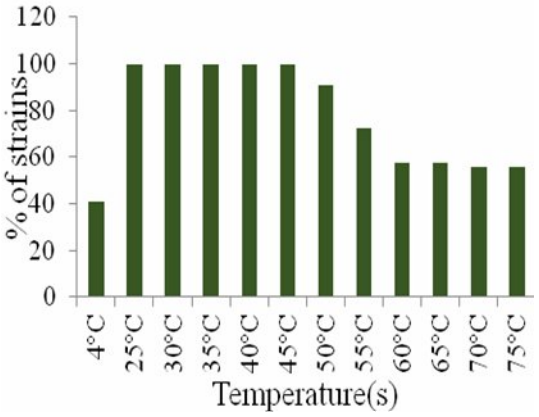

Fig. 1: Temperature defined growth profile of the isolates.

For the zymogenic screening of large number of isolates (particularly for extracellular enzymes), plate screening method is most commonly adapted owing to being less expensive, and for producing easy-to-interpret visual results [9]. All the isolates were screened for their potential to produce enzymes such as amylase, caseinase, gelatinase, and keratinase by adding respective substrates in agar medium plates. It was observed that only 7.4% of the isolates were able to hydrolyze all the tested substrates i.e. casein, gelatin, keratin, and amylase. Proportion of gelatinase producers and caseinase producers was found as 37% and 33% respectively. Out of 54 strains, 07 strains (12%) were able to hydrolyze keratin and starch as a substrate (fig. 2a & b). Interestingly, all the keratinolytic strains were found to be caseinolytic and gelatinolytic as well. Since, keratinases are the proteases with broad substrate specificities; they can hydrolyze both soluble and insoluble proteinaceous substrates such as stratum corneum, bovine serum albumin, casein, azo-keratin, gelatin, feathers, haemoglobin, wool, nails, silk, elastin, collagen, hair, and horn etc [24, 25].

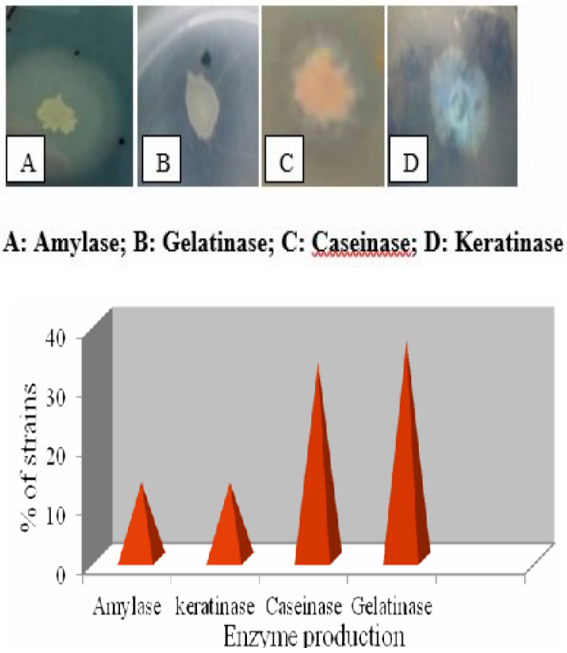

Fig. 2: (a& b) Zymogenic screening of the isolated strains using agar plates incorporated with respective substrates.

Among the 07 keratinase producers, strains designated as MW45 and MW48 were selected for further studies since both of these were found capable of complete feather degradation within 72 h (fig. 3a).

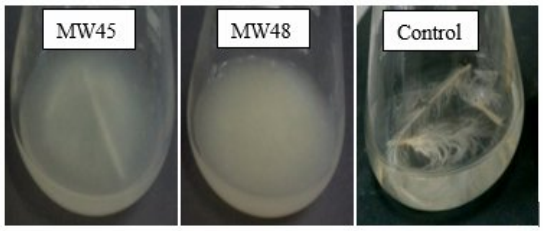

Fig. 3a: Complete feather degradation by the selected keratinolytic strains (MW45 & MW48).

A number of factors such as incubation time, temperature, pH, aeration, and composition of the fermentation medium play a very crucial role in enzyme production thus, optimization of these physicochemical factors sounds much logical to enhance the yield [26]. Studies revealed that supplementation of some nitrogen and carbon sources (tryptone, urea, peptone, yeast extract) results in

increased keratinase titers [27]. Results found in this study are in conformity with Lakshmi *et al.,* (2013) who reported that higher yield of keratinase enzyme is supported by complex medium such as Luria broth and nutrient broth [23].

As shown in fig 3b, Keratinase production from both the producer strains was also found maximum after 72 h with complete feather degradation. Similar results were found by Sivakumar *et al.*. (2011) with a strain of *Bacillus cereus* [22]. Lakshmi *et al.,* (2013) also reported maximum keratinase production from strains BF11 and BF21 in late exponential phase of growth along with complete feather degradation [23].

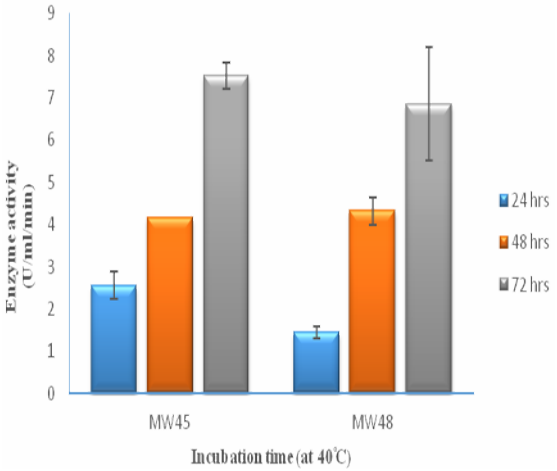

Fig. 3b: Effect of incubation time on the production of Keratinase enzyme(s) from selected strains.

When different substrates were compared such as soluble azo-keratin and native chicken feathers, enzyme production was found highest with native feathers from both the producer strains at 45°C. Whereas, keratinase production was found negligible in the presence of soluble keratin (fig. 4a and 4b). Keratinase is an inducible enzyme i.e. its production is subjected to the availability of substrates. Our results are analogous with the findings of Riffel and Brandelli (2006), who also observed the highest keratinase production in the presence of feathers [2]. This may be because of the reason that keratin degradation and keratinase production depend on cultural conditions, different kinds of substrates, and species of micro-organisms [28].

As depicted in fig 4a and b, keratinase production was found maximum at temperature range of 40 to 45°C for both the potential strains, while at 55°C, both the isolates were found unable to produce keratinase enzyme (fig. 4a and 4b). These findings are in line with the findings of Allure *et al.,* (2015) who documented 45°C as optimum temperature for keratinase production by *Streptomyces minutiscleroticus* [29]. In contrast, Cai *et al.*. (2008) found 55°C as optimum for maximum keratinase production by *Bacillus* species [30].

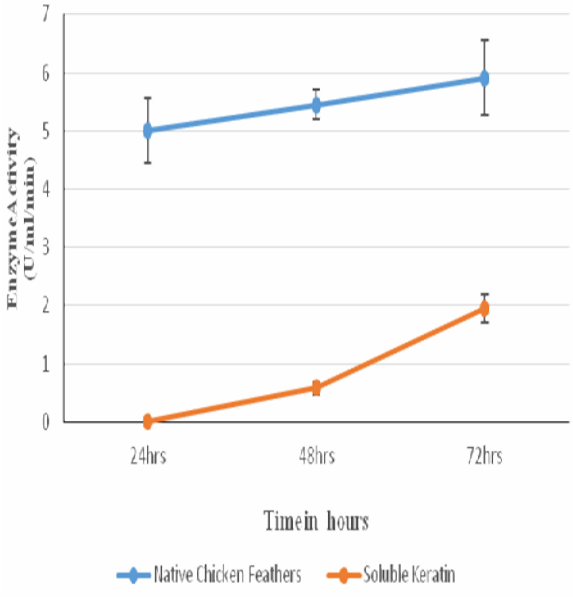

Fig. 4a: Effect of substrate on the production of Keratinase enzyme from MW45 strain

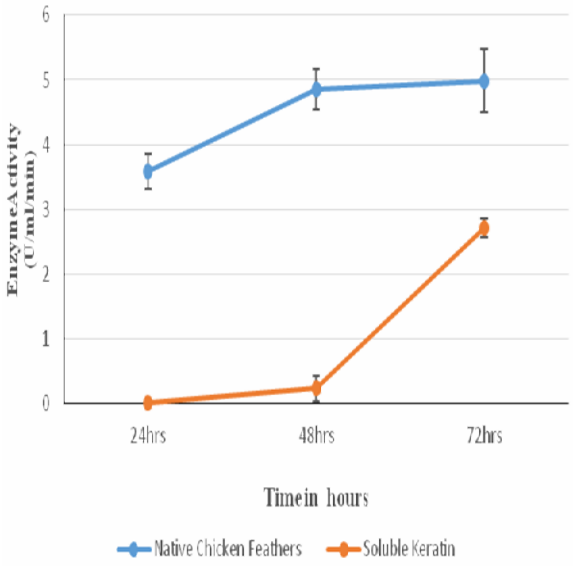

Fig. 4b: Effect of substrate on the production of Keratinase enzyme from MW48 strain.

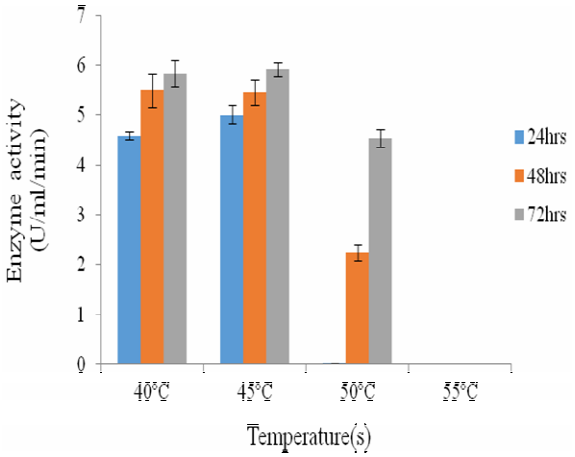

Fig. 5: (a) Effect of temperature on Keratinase production from MW45 strain

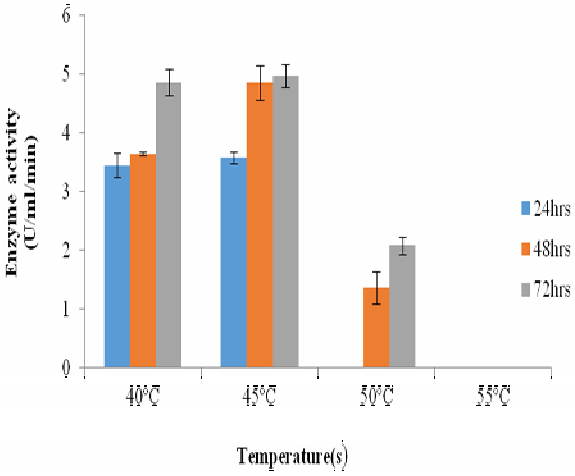

Fig. 5: (b) Effect of temperature on the Keratinase production from MW48 strain

It is reported that gene expression of proteolytic enzymes is pH regulated [31]. As per observed results, production of keratinase enzyme(s) from both the strains was not much affected at a varied pH range i.e. 4.0-11. However, maximum feather degradation was observed at pH 9.0 (fig. 6a & b). It is reported that alkaline pH favors keratin degradation as cysteine residues are modified to lanthionine making it vulnerable for hydrolytic action. A number of studies reported keratinase pH optima in alkaline range and presented these keratinolytic enzymes as favorable candidates for industrial applications [32, 33]. However, some fungal proteases are released as pre-proteins and require acidic pH to undergo auto-proteolysis of a peptide fragment that leads to the synthesis of mature proteases [34].

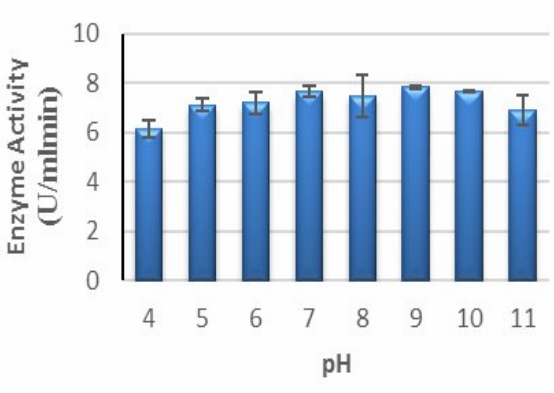

Fig. 6: (a) Effect of pH on the production of Keratinase enzyme from MW45 strain.

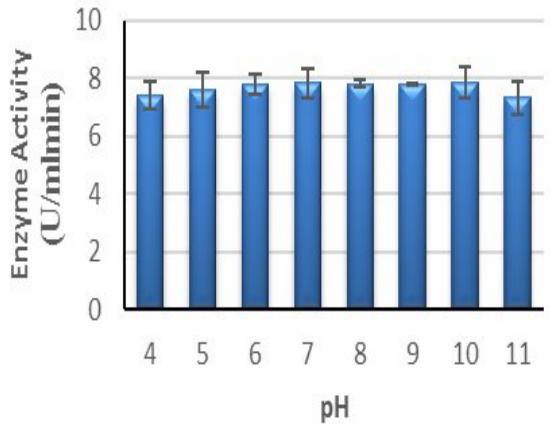

Fig. 6b: Effect of pH on the production of Keratinase enzyme from MW48 strain.

When the effect of temperature on the activity of keratinase enzyme(s) was analyzed, maximum enzyme activity was found at 60°C for the strain MW45, and 70°C for the strain MW48 (fig. 7a and 7b). When the time-temperature relationship was explored at the enzyme's optimum temperature, it was noticed that the keratinase from MW45 was active at 60°C for an hour and there was no significant decrease in the enzyme's activity. Whereas, a slight decrease in activity was observed after 40 minutes in case of keratinase enzyme from MW48 strain when it was incubated at 70°C for an hour. Our results are in accordance with Vigneshwaran *et al.,* (2010) who reported maximum activity of keratinase at 60°C by *Bacillus licheniformis* [35]. In contrast, the optimum activity of keratinase enzyme was found 45°C and 55°C by Syed *et al.,* (2009) and Cai *et al.,* (2008) respectively [13, 30].

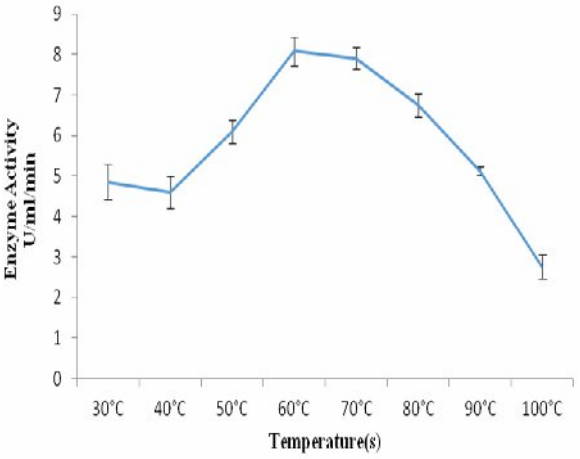

Fig. 7: (a) Effect of temperature on the activity of Keratinase enzyme from MW45 strain

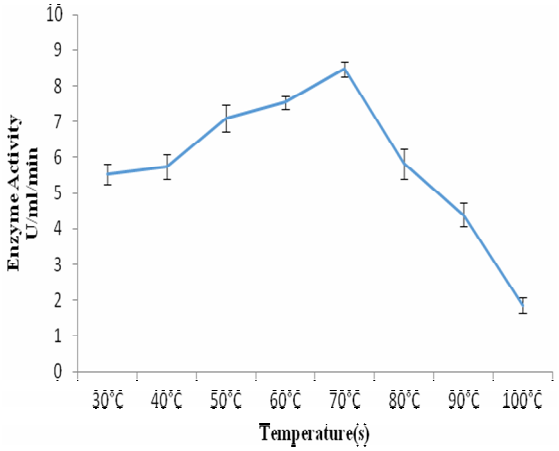

Fig. 7: (b) Effect of temperature on the activity of Keratinase enzyme from MW48 strain

Conclusion

The present study explored the potential of indigenous bacterial strains (isolated from poultry farm soil) for the production of an array of industrially important extracellular enzymes such as amylase, caseinase, gelatinase, and keratinase. Two of these strains i.e. MW45 and MW48 showed potential to hydrolyze native chicken feathers completely within 72 hrs of incubation, that can be further exploited in poultry waste management strategies as an environment friendly approach. The keratinase(s) characterized in the current study were also found active at higher temperatures (60^{o}C to 70^{o}C) suggesting their potential role in a number of industrial applications.

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