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Research Article

Optimizing the Fermentation Conditions and Enhancing the Keratinase Production from Streptomyces coelicoflavus

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Abstract

Total eight soil samples were taken from Sangli district (Maharashtra) India. The potent isolates was identified by using morphological, cultural, biochemical, physiological and 16S rRNA gene sequence analysis. Keratinase production was optimized using different parameters. The 120 hrs incubation period of was observed for optimum keratinolytic protease production**.** Inoculum age of 10 days was found to be effective in terms of protease production and degradation. 3% inoculum size was showed optimum degradation and proteolytic activity. The agitation rate at 160 rpm was found to be optimum. Optimum enzyme production and feather degradation was found at pH 9.0. Maximum production of protease was observed at 40^0C. The optimum amount of keratinase was produced by *Streptomyces coelicoflavus* in presence of dextrose and peptone as a carbon and nitrogen source separately respectively. 1% feather concentration was found optimum keratinase production. *Streptomyces coelicoflavus* was enhanced feather degradation in the presence of KH_2PO_4.

Keywords: *Streptomyces coelicoflavus*, 16S rRNA, Keratinase, Optimization and Degradation.

Introduction

Keratin protein is an structural and insoluble protein present in of vertebrates (specifically epithelial cells) and the major constituents of skin and its attachments like as hair, feathers, nail, wool, hooves, beaks, and stratum corneum [1], which have high mechanical stability and not degrading easily by most commonly proteolytic enzymes like trypsin, pepsin, and papain [2]. Because of presence of disulfide bonds in keratin protein [3,4].

Proteases is very important group and are used extensively in various industries such as leather, textiles, detergents, cheese, meat tenderization, baking, dehairing, brewery, organic synthesis and waste water treatment [5]. Keratinase enzymes have ability to recover of silver ions from used photographic film and in digestive aids [6].

Keratinase enzymes are capable of degrading keratins, which is recalcitrant proteins. Keratinases secreted mostly from genus *Bacillus, Aspergillus, Actinobacteria*, and soil microorganisms have reported for effective degradation of keratinous waste [7].

In fermentation process, keratinase production by microbes is enhanced by number of factors such as time period, inoculums age, inoculums size, aeration, pH and temperature, different nutrient source such as carbon, nitrogen, feather concentration and phosphate sources. All the above factors are used for optimization of culture and medium composition to maximize degradation of the keratin waste and increase the enzyme yield [8,9]. All factors have varied effects on different species. The keratinase production varies from organism to organism. The nutritional and environmental factors controlling the keratinase production [10].

Our main attention is focused on microbial culture and keratinase enzyme optimization. Keratinase enzymes have potential application of bioconversion of keratin wastes from poultry and leather industries. Keratinase enzymes use for the development of economically and environment friendly processes.

Material and Methods

Isolation, screening and identification

Total eight soil samples were collected in sterile polythene bags from poultry waste dumping sites and slaughter house sites from Shirala, Walawa and Miraj taluka presently situated in Sangli district. One gram of all eight soil samples were added separately in feather basal salt medium for enrichment. After 10-12 days, each enriched samples were diluted up to 10^6 by using serial dilution method. Each 0.1ml enriched diluted samples tube was removed and add on sterile glycerol aspargine agar medium (contained: L-aspargine-0.1g, K_2HPO_4-0.1g, glycerol-1g,trace salt solution-0.1ml, distil water-100ml, agar-2.5gm and pH-7.4.) [11], separately. Actinobacteria were isolated by using spread plate technique from soil samples. The isolated Actinobacteria were proceeding for primary and secondary screening using sterile milk agar medium (Contained: peptone, 0.5, yeast extract, 0.3, dextrose, 0.1, skimmed milk, 1 ml, agar 2.5 gm and pH 7.2 distil water-100 ml.) and feather meal salt agar medium (raw chicken feathers , 1g; $MgSO_4$, $7H_2O$ 0.02 g; K_2HPO_4 0.03 g; KH_2PO_4 0.04 g; $CaCl_2$ 0.022 g and Yeast extract 0.01 g and distil water-100 ml.) respectively. In order to studied morphological, biochemical and physiological characteristics of potential Actinobacteria [7]. 16S rRNA sequencing technique was used for identification of potential Actinobacteria. Name of the primer used for forward sequencing was 27F with sequence details AGAGTTTGATCMTGGCTCAG having number of base 20. Name of the primer used for reverse sequencing was 1492R with sequence details TACGGYTACCTTGTTACGACTT having number of base 22. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The phylogeny analysis of sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment [12].

Fermentation optimization studies for enhanced feather degradation

Enhanced feather degradation processes was study using physical and chemical factors like fermentation time period, inoculums age, inoculums size, aeration, pH and temperature, different carbon, nitrogen , phosphate sources and feather concentration.

Effect of incubation period on fermentation

The effect of incubation period on feather degradation by *S. coelicoflavus* was grown on basal salt medium containing 3% feathers. *S. coelicoflavus* inoculated in sterile basal feather medium flasks were incubated for period of 10 days at 40 (±2) °C on rotary shaker at 100 rpm. The pH of the medium was adjusted to 8. During the incubation, protease activity was estimated at every 24 hrs [13]. An uninoculated flask with feather was maintained as controls throughout the experiments.

Effect of inoculum age on fermentation

The culture of *S. coelicoflavus* was use to determine the effect of inoculum age on feather degradation. Inoculum was prepared with different ages 7 to 12 days with 24 hrs. inoculums age increments. The inoculum was added to sterile basal feather medium flasks and incubated up to 14d fermentation period. Protease activity was examined as per standard assay method [12]. An uninoculated flask with feather was maintained as controls throughout the experiments.

Effect of inoculum size on fermentation

To determine the effect of inoculum size on feather degradation, prepared *S. coelicoflavus* inoculum was added at different concentrated of volume (1.0 to 5.0 % v/v) at 1.0 % increment to sterile basal feather medium flasks and incubated till the end of fermentation period at 40^0C. At the end of incubation time the supernatant was examined for protease activity [14].

Effect of aeration (agitation rate) on fermentation

The culture of *S. coelicoflavus* was grown in basal salt feather medium on a rotary shaker at different agitation rate from 100-200 rpm with 20 rpm increment and incubated at 40^0C for 12 days. At the end of fermentation process the supernatant was removed from flask for studying protease activity [15].

Effect of pH on fermentation

The *S. coelicoflavus* culture was inoculated in basal salt feather medium with different pH range 6-10 with pH 1.0 increment for 8 days at 40^0C on rotary shaker at 160 rpm. After incubation, visually degradation of feathers was observed at the end of fermentation time Protease activity was estimated using cell free supernatant [15].

Effect of temperature on fermentation

To study keratinolytic enzyme production with optimum temperature for feather degradation by culture *S. coelicoflavus* was grown in basal feather medium under shaking at different temperatures range 25-60°C with 5°C interval. Supernatant was examined for alkaline protease activity [16].

Effect of chemical parameters

Effect of different carbon sources on fermentation

To study the effect various carbon sources on culture conditions for the keratinolytic enzyme production by *S. coelicoflavus*. Different carbon sources such as xylose, dextrose, sucrose, mannitol and starch were supplemented separately to the basal feather medium containing 1 % chicken feather. All carbon sources were added in the medium at 0.1 (% w/v) concentrations [16].

Effect of different nitrogen sources on fermentation

To study effect of various nitrogen sources on culture conditions for the keratinolytic enzyme production by *S. coelicoflavus*. There are five nitrogen sources such as peptone, tryptone, beef extract, potassium nitrate and ammonium sulfate were used separately to the basal feather medium containing 1% chicken feather. 0.01% (w/v) concentration of all nitrogen sources were presented in the medium [16].

Effect of feather concentration on fermentation

In order to study the substrate concentration for influence feather degradation by *S. coelicoflavus*. The *S. coelicoflavus* isolates was grown in the basal feather medium supplemented with different concentrations of feather (0.5-4% with 0.5% increment) separately and incubated for 12 days at 40 (±2)°C [15].

Effect of different phosphates on fermentation

The influence of feather degradation in presence of phosphates by *S. coelicoflavus* was studied. The isolates was inoculated in the basal feather medium containing various phosphates sources such as Potassium di-hydrogen phosphate, di-potassium hydrogen phosphate, Sodium di-hydrogen phosphate and Ammonium phosphate at 0.1 % (w/v) concentration separately and incubated for 10 days at 40 (±2) °C [15].

Results

Sr. No.	Morphological characteristics	Isolates *S. coelicoflavus*
1	Color of Arial Mycelium	Discrete
2	Color of Substrate Mycelium	Reddish
3	Spore chain morphology	Rectiflexibiles
4	Pigment production	Red

Table 1: Morphological characteristics of *S. coelicoflavus* isolates.

Sr. No.	Media Isolates	Characters	NA	GAA	TYEA	PYEIA	ISSA	TA	SCA
1	*S. coelicoflavus*	Growth	+++	+++	++	+++	++	+	+++
		Aerial mycelium	√	√	√	√	√	√	√
		Substrate mycelium	√	√	√	√	√	√	√
		Pigmentation	Nil	Nil	Nil	Nil	√	Nil	√

Table 2: Cultural characteristics of isolates on different media.

Where, NA: Nutrient Agar; GAA: Glycerol Aspargine Agar; PYEIA: Peptone Yeast Extract Iron Agar; SCA: Starch Casein Agar; ISSA: Inorganic Starch Salt Agar; TYEA: Tryptone Yeast Extract Agar; And TA: Tyrosine Agar.

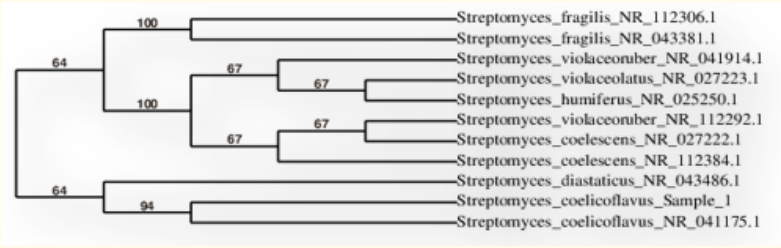

Figure 1: Phylogenetic analysis of 16S rRNA gene of isolate S. coelicoflavus by neighbour joining method.

Sr. No.	Characteristic		Result
1	Carbon utilization	Glucose	AG
		Sucrose	A
		Mannitol	AG
		Xylose	AG
		Arabinose	AG
		Lactose	-
		Na-citrate	+
		Na-acetate	+
2	Nitrogen utilization	L-phenylalanine	+
		L-Cysteine	-
		L-Histidine	+
		DL-Valine	+
		L-Tyrosine	+
3	Enzyme activity	Catalase	+
		Oxidase	+
		Lecithinase	+
		Lipolysis	+
		Protease	+
		Nitrate reductase	+
		Gelatinase	+
		Amylase	+
		Urease	+
		H_2S production	-
4	Effect of Temperatures on growth	10^0C	-
		20^0C	+
		30^0C	+
		40^0C	+
		50^0C	+
5	Effect of pH on growth	4	+
		6	+
		8	+
		10	+
6	Growth in presence of inhibitory compounds	Crystal violet (0.0001%)	-
		Phenol (0.1%)	-
		Sodium azide 0.001%	+
		Sodium azide 0.002%	-
		Sodium chloride (NaCl) 1%	+
		Sodium chloride (NaCl) 4%	+
		Sodium chloride (NaCl) 7%	-

Table 3: Biochemical and physiological characteristics of *S. coelicoflavus* isolates.

Where, A: Acid; AG: Acid with Gas; +-Positive and -Negative

According to morphological, cultural, biochemical, physiological and 16S rRNA sequencing analysis isolates was identified as a *S. coelicoflavus.* The optimization of fermentation condition was studied for *S. coelicoflavus.*

The sequence of isolate was deposited to DDBJ database for annotation and accession numbers of isolates was obtained (Table 4).

Sr. No.	Actinobacteria	Accession numbers by DDBJ
1	*Streptomyces coelicoflavus*	LC072737

Table 4: Accession numbers of identified *Streptomyces spp.*

Effect of incubation period on fermentation

The effect of period on fermentation process was studied. It is observed from table and graph that isolates showed optimum enzyme activity at 168 hrs (56.03 U/ml). Minimum enzyme activity of isolates was observed at 24hrs. It is clear from this table and graph, 7 days its optimum period for fermentation process**.** The enzyme level reached the maximal limit by 168 h and further exhibited a gradual decline.

Effect of inoculum age on fermentation

Enzyme production was studied by *S. coelicoflavus* after inoculated with different inoculum ages of 24, 48, 72, 96, 120, 144, 168 and 192 h old inoculum. Optimum enzyme production was observed by *S. coelicoflavus* at inoculums age of 144 hrs old, where protease activity was about 53.40 U/ml. Minimum protease production (10.12 U/ml) was recorded when the inoculum age was 24 h old.

Sr. No.	Fermentation period (Hrs.)	Enzyme activity (U/ml)
1	24	1.1
2	48	5.2
3	72	9.6
4	96	14.2
5	120	20.3
6	144	34.01
7	168	56.03
8	192	48.5
9	216	42.1
10	240	38.2
11	Control	00.0

Table 5: Effect of fermentation period on fermentation by *S. coelicoflavus.*

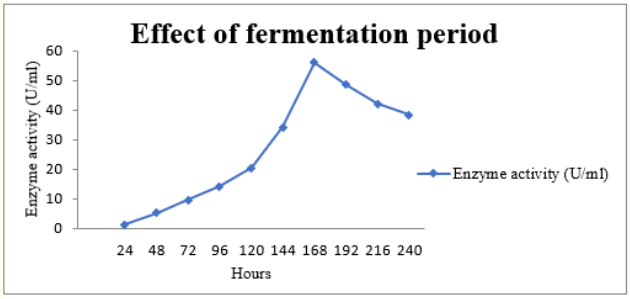

Figure 2: Effect of fermentation period on fermentation by *S. coelicoflavus.*

Sr. No.	Inoculum age (Hrs)	Enzyme activity (U/ml)
1	24	10.12
2	48	18.62
3	72	24.23
4	96	31.27
5	120	39.00
6	144	53.40
7	168	51.53
8	192	48.54
9	Control	0.0

Table 6: Effect of inoculums age on fermentation by *S. coelicoflavus.*

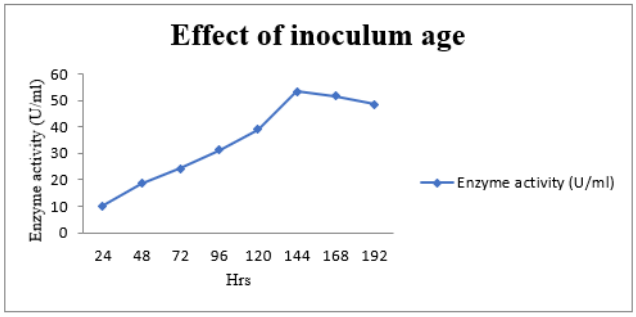

Figure 3: Effect of inoculum age on fermentation by *S. coelicoflavus.*

Effect of inoculum size on fermentation

Optimum keratinase enzyme production was observed at 3% inoculums size. The keratinase activity was showed 55.3 U/ml at 3% inoculums size.

Sr. No.	Inoculum size (%)	Enzyme activity (U/ml)
1	0.5	8.2
2	1	13.8
3	1.5	21.25
4	2	29.20
5	2.5	41.12
6	3	55.3
7	3.5	49.1
8	4	37.26
9	4.5	26.14
10	Control	0.0

Table 7: Effect of inoculums size on fermentation by *S. coelicoflavus.*

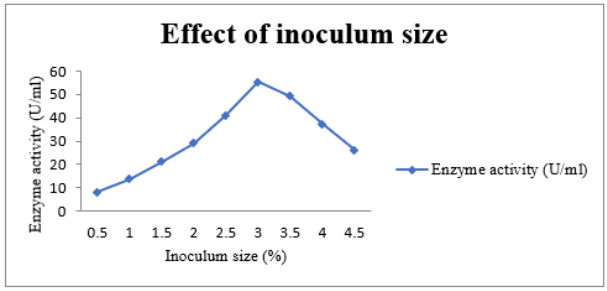

Figure 4: Effect of inoculum size on fermentation by *S.coelicoflavus.*

Higher the inolulum size, lower the production of keratinase enzyme was observed. Hence the in coulum size of 3% of the test strain was employed in further studies.

Effect of aeration (agitation rate) on fermentation

Effect of aeration was studied. It is clear from the table and graph optimum keratinase enzyme production was recorded at shaking speed of 160rpm (56.0 U/ml). However, further increase in the shaking speed did not influence the production of keratinase enzymes beyond 160 rpm shaking. Hence, the agitation rate of 160 rpm was used in all the further studies.

Effect of fermentation medium pH on fermentation by *S. coelicoflavus*

Effect of medium pH on fermentation condition was determined keratinase activity in terms of U/ml. Keratinase activity of *S. coelicoflavus* in range of pH 6 to 10 was found from 37.23 to 54.10 U/ml.

Maximum keratinase activity in terms of U/ml of *S. coelicoflavus* was observed at pH 8 and minimum keratinase activity in terms of U/ml of *S. coelicoflavus* was observed at pH 6. Thus pH 8 was found optimum for enzyme activity of *S. coelicoflavus.* Also the maximum degradation of feather was visual observed in basal feather medium (pH 8.0) inoculated with *S. coelicoflavus* after comparison with control.

Sr. No.	Aeration rate (rpm)	Enzyme activity (U/ml)
1	100	32.11
2	120	40.18
3	140	48.43
4	160	58.54
5	180	50.12
6	200	42.10
7	Control	0.0

Table 8: Effect of agitation rate on fermentation by *S. coelicoflavus.*

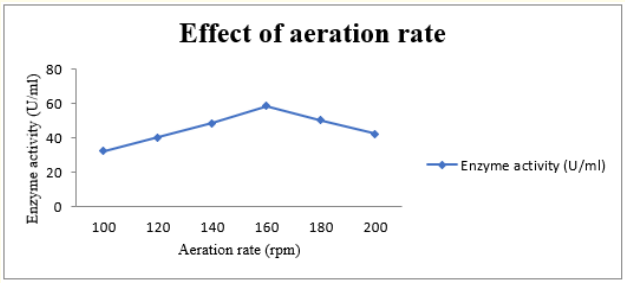

Figure 5: Effect of agitation rate on fermentation by *S. coelicoflavus.*

Sr. No.	pH	Enzyme activity (U/ml)
1	6	37.23
2	7	45.60
3	8	54.10
4	9	51.20
5	10	46.40
6	Control	00.00

Table 9: Effect of fermentation medium pH on fermentation by *S. coelicoflavus.*

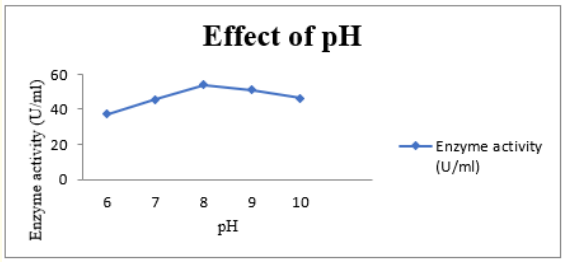

Figure 6: Effect of fermentation medium pH on fermentation by *S. coelicoflavus*.

Effect of fermentation temperature on fermentation

In order to determine the optimum temperature for keratinase production, of *S. coelicoflavus* was grown in basal feather medium. No significant enzyme production was found at temperature 25 °C. Maximum keratinase enzyme activity in terms of U/ml of *S. coelicoflavus* was observed at temperature 40°C (54.5 U/ml) and minimum keratinase activity in terms of U/ml of *S. coelicoflavus* was observed at 25 °C. Thus 40 °C was found optimum temperature for keratinase activity.

Effect of carbon source on fermentation by *S. coelicoflavus*

The result on the ability of *S. coelicoflavus* on protease production by utilizing various carbon sources are given in table 11.

Among the carbon sources it was observed dextrose significantly influenced of keratinase production (54.12U/ml). The concentration of dextrose for the maximum production of keratinase was 1%. *S. coelicoflavus* produces less keratinase (16.3 U/ml) by utilizing mannitol.

Sr. No.	Temperature ^{0}C	Enzyme activity (U/ml)
1	25	26.10
2	30	34.23
3	35	43.15
4	40	54.5
5	45	52.32
6	50	48.24
7	55	42.14
8	60	36.26
9	Control	00.0

Table 10: Effect of temperature on fermentation by *S. coelicoflavus.*

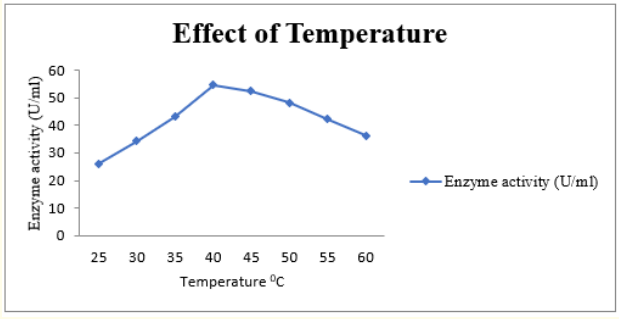

Figure 7: Effect of temperature on fermentation by *S. coelicoflavus*.

Sr. No.	Carbon source	Enzyme activity (U/ml)
1	Dextrose	54.12
2	Mannitol	16.3
3	Sucrose	34.15
4	Starch	40.14
5	Xylose	36.6
6	Control	00.0

Table 11: Effect of carbon source on fermentation by *S. coelicoflavus.*

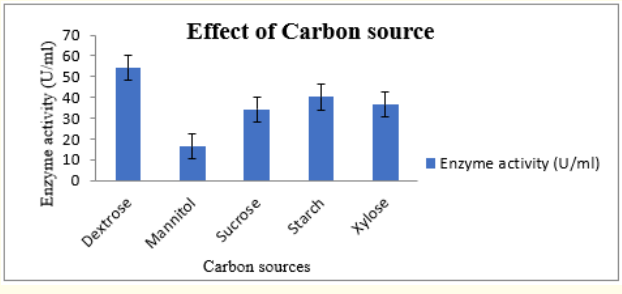

Figure 8: Effect of carbon source on fermentation by *S. coelicoflavus*.

Effect of nitrogen source on fermentation by *S. coelicoflavus*

The maximum amount of keratinase (63.10U/ml) produced by *S. coelicoflavus* was observed with peptone and minimum amount of keratinase (20.27 U/ml) produced by *S. coelicoflavus* was observed with potassium nitrate.

Effect of feather concentration on fermentation by *S. coelicoflavus*

In order to determine the maximum amount of keratinase enzyme (56. 22U/ml) produced by *S. coelicoflavus* was observed with feather concentration. Among various concentrations of feather, 1% (w/v) significantly enhanced keratinase production. No significant enzyme production was found at 3% feather concentration. Thus 1% feather was found optimum concentration for keratinase activity.

Sr. No.	Nitrogen source	Enzyme activity (U/ml)
1	Peptone	63.10
2	Tryphtone	52.24
3	Beef extract	34.26
4	Ammonium Sulphate	33.65
5	Potassium Nitrate	20.27
6	Control	00.0

Table 12: Effect of nitrogen source on fermentation by *S. coelicoflavus.*

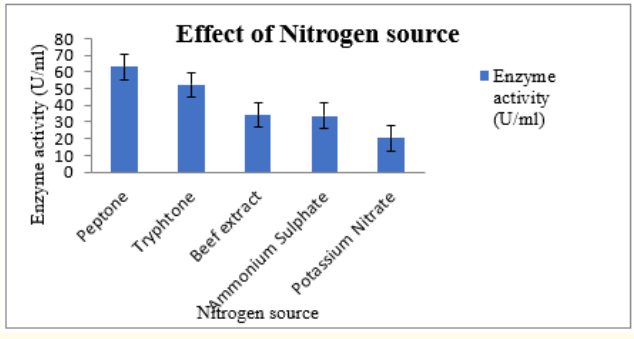

Figure 9: Effect of nitrogen source on fermentation by *S. coelicoflavus*.

Sr. No.	Feather concentration (%)	Enzyme activity (U/ml)
1	0.5	39.11
2	1.0	56.22
3	1.5	46.12
4	2.0	34.14
5	2.5	20.21
6	3.0	9.62
7	3.5	6.01
8	4.0	1.23
9	Control	00.0

Table 13: Effect of feather concentration on fermentation by *S. coelicoflavus.*

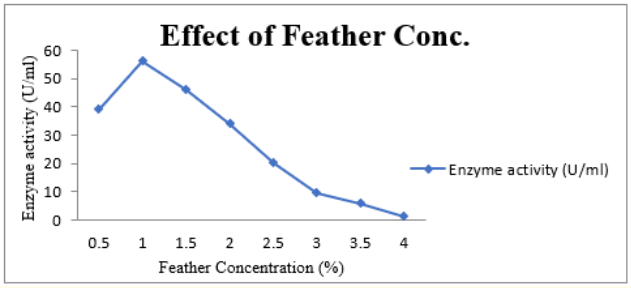

Figure 10: Effect of feather concentration on fermentation by *S. coelicoflavus*.

Effect of phosphate source on fermentation by *S. coelicoflavus*

The effect of different phosphates on enzyme production has been shown in figure and table 14. Maximum keratinase production (57.14U/ml) observed with Potassium dihydrogen phosphate was supplemented. Ammonium phosphate had a moderate positive influence on the keratinase activity.

Sr. No.	Phosphate source	Enzyme activity (U/ml)
1	Potassium di-hydrogen phosphate (KH_2PO_4)	57.14
2	Di-potassium hydrogen phosphate (K_2HPO_4)	46.21
3	Sodium di-hydrogen phosphate (NaH_2PO_4)	49.54
4	Ammonium phosphate (Na_2HPO_4)	51.36
5	Control	00.00

Table 14: Effect of phosphate source on fermentation by *S. coelicoflavus.*

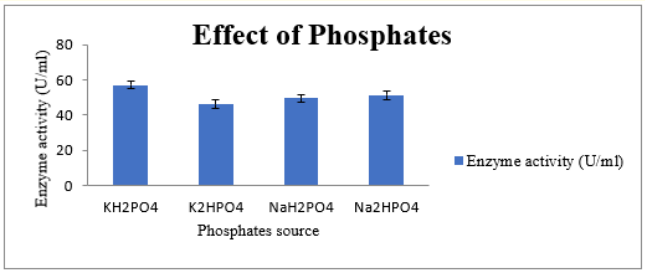

Figure 11: Effect of phosphate source on fermentation by *S. coelicoflavus*.

Discussion

The optimization of culture conditions and medium compositions for keratinase production by micro-organisms is very important. Actinobacteria produce various secondary metabolites including enzymes. Actinobacteria strains were isolated and screened for keratinase. Several researchers were used different screening media for the determination of keratinase [7]. The isolates showed potent keratinase activity on feather meal agar medium plate. It showed zone of hydrolysis and isolates was found to be high to other isolates.

Based on these morphological, cultural, physiological and molecular characteristics the selected actinomycete was identified as *Streptomyces coelicoflavus* [12]. The fermentation process parameters were optimized to enhance the production of keratinase enzyme. As per our result keratinase production was found to be high after 168 hrs (7th day) of incubation at 40^{0}C. The similar results also observed in the study using the other Actinobacteria, especially *Streptomyces rimosus* [17] and *Streptomyces cyanens* [18]. Saha., *et al.* [19] reported the optimum enzyme activity was observed between 16th to 19th days from the day of incubation for the four experimental *Nocardiopsis* sp. The conclusion was supported partly by our work. The results of the present finding on fermentation period confirmed the synthesis of keratinase at the end of log phase.

The fermentation medium optimum keratinase enzyme production was observed at 3% inoculums size. Further increased the concentration of inoculums valume cause gradual decreased keratinase enzyme activity. The similar results also shown by Sivakumar., *et al.* [20] as the inoculum volume was further increased, the production of keratinase enzyme was gradually decreased. The reduction in keratinase yield after the optimum period was probably decreased synthesis of enzymes [21]. Similar findings have been reported also by other workers [22,23].

In our study, isolates *Streptomyces coelicoflavus* was produced maximum keratinase (58.54U/ml) at 160 rpm. The fermentation medium was provided higher agitation rates (200 rpm) observed good growth of bacteria but low keratinase production because high DO concentration. The similar results also appeared in the study using the both isolates produced maximum keratinase (57.33 ± 2.08) U/mL for *B. cereus* and (38.00 ± 3.00) U/mL for *Pseudomonas sp*. at 150 r/min (Mursheda Akhter., *et al.* 2020).

In fermentation process, the optimal pH of medium for keratinase enzyme production (54.10 U/ml) was observed at pH 8.0. Nadeem., *et al.* [24] reported similar results that the pH value 8.0 enhanced the production of alkaline keratinase enzyme by *Streptomyces pulvereceus* MTCC 8374.

Temperature is one of the important and significant factors to influenced alkaline keratinase production. Optimum enzyme production was showed at 40^0C. The similar results by Nayaka and Vidyasagar (2013) reported serine protease from *Streptomyces spp.* and found their optimally activity at 40^0C. The mostly keratinase enzyme was observed thermo active nature in the range of 50°C to 60°C, Lateef., *et al.* [2] reported that *B cereus* LAU shows the highest keratinase activity at 50°C whereas the maximum keratinase activity found for *Microbacterium sp.* at 55°C [25].

In this study, carbon source such as dextrose was showed enhanced keratinase enzyme production. Our result was supported strongly by Kumar., *et al.* (2008). Sen and Satyanarayana (1993) were reported that the carbon sources such as fructose and lactose enhanced keratinase production. Some researchers reported glucose was showed negative effect on keratinase production, for example *A. fumigatus* [26], *Stenotrophomonas sp.* D-1 [27].

In our investigation, supplementation of peptone greatly elevated the keratinas production for *S. coelicoflaus*. The similar results also appeared in the study using the bacterium *Stenotrophomonas Maltophilia* by Kumar., *et al.* [10]. Supplemented with Yeast extract was enhanced keratinase production from *Streptomyces sp.* [17].

After optimization, it is clear that, additional carbon and nitrogen sources have stimulatory effect on keratinase production.

The supplement phosphate source such as Potassium di-hydrogen phosphate was showed enhance the keratinase production. Similar result was reported Jeong., *et al.* [28].

Optimum keratinase production was depended on feather concentration. When the feather concentration level was increased beyond 1 % (w/v), keratinase production showed slightly decreased. Jain., *et al.* [14] was reported varied feather concentrations from 0.5-2% using *Streptomyces exfoliatus* CFS1068 strain; the maximum yield keratinase observed in the flasks containing 1% feather [29-31].

Conclusion

Extracellular alkaline keratinase producing *Streptomyces coelicoflaus* was isolated, screened, characterized and identified. The fermentation process parameters were optimized to enhance the chicken feather degradation. Enzyme obtained from *S. coelicoflaus* hydrolyzed chicken feather, will help to treat this waste. This keratinolytic *S. coelicoflaus* strain and enzymes may be used for different industrial applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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