Int.J.Curr.Microbiol.App.Sci (2016) *5*(7): 153-163

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 7 (2016) pp. 153-163**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.507.015

Isolation, Identification and Characterization of Keratinolytic *Streptomyces coelicoflavus*

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Keywords

Actinomycetes, Streptomyces, Keratinase , Feather meal.

Article Info

Accepted:
08 June 2016
Available Online:
10 July 2016

A B S T R A C T

Fourteen isolates were obtained from poultry and beam house region soils of Sangli district. An isolate showing maximum keratinolysis was identified as *Streptomyces coelicoflavus.* The ability of *Streptomyces coelicoflavus* to utilize chicken feather as a substrate was tested. Crude extract of keratinase enzyme from feather meal broth was used for characterization. It was found that maximum enzyme activitywas49U/ml. Crude keratinase was optimally active in the pH range from 7.5 to 8 and at temperatures from 35^0C to40^0C. The various divalent metal ion were tested, Ca^{++} and Zn^{++} were found to enhance the activity whereas Mg^{++}, Cu^{++}, Mn^{++} inhibit the enzyme activity. Also reducing agents and inhibitors were tested. Reducing agent such as Dithiothreitol (DTT) inhibit the enzyme activity whereas sodium sulphide, SDS increases the enzyme activity. 1,10phenanthroline and EDTA were increases the enzyme activity.

Introduction

Feather is almost pure keratin protein consisting of amino acids (Mukesh kumar, 2012). In nature, keratin occurs mainly in the form of hair, horn, nail and cornified tissue. Worldwide about 24 billion chickens are killed annually and totally about 8.5 billiontonnes of poultry feather are produced (Agrahari, 2010). According to recent report India's contribution alone is about 250 million tonnos (Agrahari, 2010).

Feather contains over 90% protein. The main component being β-keratin, a fibrous and insoluble structural protein extensively crossed-linked with disulphide bond, hydrogen bond, hydrophobic interaction, resulting in the mechanical stability, of keratin and resistance to common proteolytic enzyme like as pepsin, trypsin and papain (Onifad *et al.*, 1998). Proteases constitute one of the most important groups of enzyme and their annual sales account for

60% of the world enzyme market (Turk 2006). Keratinase (E.C.3.4.95%) belongs to the class hydrolase which are able to hydrolyze insoluble keratins more efficient than other protease (Vigneshwaran *et al.*, 2010). Feather degrading keratinase producing bacteria are *Bacillus licheaifoemis* (Zerdani*et al.*, 2004). There are several reports that show *Streptomyces* produces keratinase enzyme e.g. *S.albidoflavus* (Bressolier*et al.*, 1999). *Streptemces spp.*, 5a4 (DeAzeredo*et al.*, 2006) and *Streptomyces* spp.,.CN 902 (Lazim*etal;* 2009). Keratinolytic activities were also demonstrated in saprophytic fungi like as *Acta Mycologia* (Kornillowicz, 1994).

In this study we identified *Streptomyces coelicoflavus* and then partially purified and characterized the secreted keratinase enzyme. The ability of this enzyme to degrade keratin based substrates selectively which was greater than the ability of other *Streptomyces spp.*

Materials and Methods

Materials

Soil sample: 8 Soil samples were collected from the sangli district M.S., India and used for isolation of *Actinomycetes.*

Glycerol aspargine broth and Glycerol aspargine agar with cycloheximed(80 µg/ml). Skimmed milk agar, Basal salt medium, Feather meal agar, Gelatine agar, Christensen's medium, Egg yolk agar, PYIA, Peptone water, Starch agar, Peptone nitrate broth, Sugar fermentation medium. Keratin solution.

Buffer: Acetate Buffer (pH- 4 to 5.5), Phosphate Buffer (pH- 6 to 7.5), Tris- HCl Buffer (pH -8 to 9).

Metal: Zn^{++}, Mg^{++}, Cu^{++},Mn^{++}, Ca^{++}. Reducinag and inhibitory agents: 1,10 Phenanthroline, SDS, EDTA, Sodium Sulphied, Dithiothreitol(DTT). Dimethyl Sulfoxide, Acetone, Trichloroacetic acid, Bradford reagent.

Methods

Isolation of *Actinomycetes*

The soil samples were collected from the villages around Shirala, Dist.-Sangli, M.S. India and enrichment of soil samples were carried out in Glycerol asparagine broth supplemented with Cycloheximide (80µg/ml). A 10-fold serial dilutions of the sample were prepared up to 10^{-6} and 0.1ml aliquots of 10^{-5} and10^{-6} dilution was inoculated into Glycerol asparagine agar (L-asparagine- 0.1g, K_2HPO_4-0.1g, glycerol-1g, trace salt solution- 0.1ml, agar- 2.5g, distilledwater-100 ml pH-7.4). To avoid the growth of fungal contaminant, medium was supplemented with Cycloheximide (80µg/ml). Plates were incubated at room temperature and monitored periodically over 5 to 7 days. Pure isolates were transferred to slants of Glycerol asparagine agar and preserved at 4°C for further study.

Identification of Isolate

Morphological characteristics were studied with cover slip culture technique. Cultural characteristics were recorded on Glycerol asparagine agar medium. Biochemical characters were recorded on the basis of sugar utilization potential, enzymatic activities and growth under inhibitory substances. On the basis of spore mass color, the substrate mycelium color, the shape of the spore chain, morphological and cultural characteristics the isolate were tentatively identified as *Streptomyces.* Biochemical characterisations of

Streptomyces producing keratinase were carried out (Williams *et al.,* 1983)

Primary Screening

The primary screening of Keratinolytic *Streptomyces* were carried on Skimmed milk agar plates (pH 6.5–7.2) containing peptone-1%, sodium chloride-0.5%, yeast extract-0.3%, agar-2% and skim milk-10%. All the plates were incubated at 30°C for 2–5 days. After incubation, the plates were observed for the zone of clearness around the colony.

Secondary Screening

Keratinolytic *Streptomyces* were screened on keratin basal salt agar. (Ghosh *et al.,* 2008).

Molecular Identification of *Streptomyces*

One of the potent keratinase producing *Streptomyces* was identified by using 16SrRNA Sequencing. Name of the primer used for forward sequencing was 27F with sequence details AGAGTTTGATCMTG GCTCAG having number of Base 20. Name of the primer used for reverse sequencing was 1492R with sequence details TACGGYTACCTTGTTACGACTT having number of Base 22. 16S rRNA gene fragment was amplified using universal primers such as above mentioned. The phylogeny analysis of sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

Preparation of Crude Enzyme

Keratinolytic *Streptomyces* colony was transfer on basal salt medium containing feather meal and kept for incubation at 35^0C for 120 to 160 hrs. with shaking at 200 rpm. The feather meal broth was mixed thoroughly with a 50ml tris-HCl buffer (50mM, pH 8). The mixture was shaken for 30min at room temperature in an orbital shaker (155strokes/min) and the process was repeated two to three times. The filtrate thus obtained was used as a crude enzyme for protease activity (Ponnuswamy *et al.,* 2012; Vigneshwaran *et al.,* 2010).

Preparation of Keratin solution

Keratinolytic activity was measured with soluble keratin (0.5% w/v) as substrate. Soluble keratin was prepared from white / black chicken feat hers by the method of Wawrzkiewick*et al.,*(1997). Native chicken feathers (10 gm) in 500 ml of dimethyl sulfoxide were heated in a hot air oven at 100^0C for 2 hrs. Soluble keratin was then precipitated by addition of cold acetone (1L) at Freezer temperature for 2 hrs, followed by centrifugation at $10,000_{Xg}$ for 10 min. The precipitate was washed two times with distilled water and dried at 40^0C in a vacuum dryer. 1gm of quantified precipitate was dissolved in 20 ml of 0.05M NaOH. The pH was adjusted to 7.0 with 0.1 M HCl and the solution was diluted to 200 ml with 0.05 mol/L Phosphate buffer(pH 7.0) (Vigneshwaran *et al.,* 2010).

Keratinase Assay

Keratinase activity was determined by the method as described by Krishna Rayudu (2014) with modification. 2ml of reaction mixture contain of 1 ml of keratin solution and 1ml crude enzyme of supernant and then reaction mixture incubated for 10 min. at 40 ^{0}Cin shaking water bath at 100 rpm agitation. 2 ml of (10%) chilled TCA was added and the mixture was kept for 20 min., followed by centrifugation 5000 rpm for 10 min. 0.1 ml of the supernant was added with 0.9 ml D/W and 0.5 ml of 500 mM sodium carbonate and incubated 35^0C for 10 min. 2

ml Folin-Ciocalteu reagent (1:3 v/v)was added and incubated for 20 min. followed by reading absorbance at 660 nm for the developed blue color. A control was processed by adding the enzyme after incubation and TCA was immediately added. A standard graph was generated using standard tyrosine solution of 10-100µg/µl. One unit of keratinolytic activity is defined as the amount of enzyme that librates 1 µg of tyrosine equivalent per min. under the described assay condition.

Protein Determination

Protein contain was measured by the method of Bradford (1976) using bovin serum albumin (BSA) as standard.

Amino acid analysis by HPLC

The method of amino acid analysis of Robert and Stephen (1984) and Jeong *et al.*,(2010) was used to determine the amino acid contents for feather degradation broth. High Performance Liquid Chromatography (HPLC) was used for amino acid analysis. In this technique quantified the amino acids by using phenylisothiocynate (PITC) as a coupling reagent. The quantitative pre column derivatization of amino acids and several reverse phase HPLC systems for separation of the resulting phenylthio-carbamyl (PTC) derivatives. During the process, 3.5µl Agilent columns (4.6 × 150 mm) were employed.

Activity of keratinase with different Parameters

Effect of pH

The optimum pH was determined at 40^0C for 1 hrs. Using following buffers (50mM/l): Sodium phosphate buffer (pH 6-7.5), Tris-HCl buffer (pH 7.5- 9).

Effect of Temperature

To determine optimum temperature for keratinolysis, enzyme reaction carried out at different temperature for 1hrs. Temperature ranging Acetate Buffer (pH- 4 to 5.5),from 30^0C to50^0C were used. Enzyme activity was determined by standard enzyme assay.

Effect of metal divalent ion

Five metal divalent ion were used to determine effect on keratinase activity, such as (5mM) Ca++, Zn++, Cu++, Mn++, Mg++ activity of metal an keratinase was determined by standard enzyme assay.

4)Effect of reducing agent and inhibitors

The effect of SDS, sodium sulphide, dithiothretol (DTT), 1,10phenanthroline and EDTA on enzymolytic activity was determined. A control was kept with the enzyme and the substrate (without above agent) and the value of the control activity was consider as 100%

Results and Discussions

Isolation of Keratinolytic *Streptomyces*

A total 14 caseinase producing isolates were screened and used in feather meal basal salt agar medium for feather degrading property it was found that the SH1strain was shown feather degradation at 30^0C within 94 hrs. The isolate SH1 strain belonging to genus *Streptomyces* was tentatively identified on the basis of morphological and biochemical characteristics were studied (Table 1).

Identification of *Streptomyces*

On the basis of morphological and biochemical characteristic (Table 1) the isolate, was identified up to the genus level

and using 16s rRNA sequence the isolates further identified up to species *Streptomyces coelicoflavus.*

Degradation of Feather

*Streptomyces coelicoflavus*LC072737 was able to grow and produce keratinase in feather meal basal salt brothmedium (Cai *et al.*,) and resulted in maximum degradation of feather after 7 days incubation at 40^0C at 150 rpm (fig.5). Keratinase activity measured was 49 U/ml in the absorbance at 660nm by standard enzyme assay method (Rayudu, 2014)

Amino acids analysis from feather degradation broth of *Streptomyces coelicoflavus* was quantified by High Performance Liquid Chromatography (HPLC). During the process Agilent columns (4.6x150mm) 3.5µl were employed.

A total 18 amino acids were found in the feather degradation broth. Cysteine was found in high concentration (3.3569µl/ml) followed byleucine (2.02558µl/ml). Total concentration of amino acid in broth was found 13.4986µl/ml. In feather degradation broth released essential amino acids such as threonine (0.4140µl/ml), methionine (1.7487µl/ml), 1-methyl histidine (0.5909µl/ml) and lysine (1.4014µl/ml). Saha (2012) and Rayudu (2014) reported 15 and 16 amino acids from feather degradation broth respectively.

Activity of keratinase with different parameters

Effect of pH

The enzyme keratinase was active in pH 6 to pH 9. Enzyme has an optimum pH 8.The enzyme activity was high for alkaline pH, similar finding was reported in *Streptomyces spp.* (Azeredo *et al.,* 2006*).* Increasing activity were reported as there is increase in pH 6 to 8 and reduced slightly from pH 8 to 9.

Effect of Temperature

The effect of temperature on keratinase catalytic activity of enzyme increased with increasing temperature up to 40^0C and beyond 40^0C reduced the enzyme activity. Similar results were also reported by Jaouadi *et al.*, in 2010 of *Streptomyces spp.*, strain AB1. Similar kind of properties were studied on *S. thermoviolaceus* (Chitte, 1999), *B. subtilis* (Setyorine *et al.*, 2006), *Streptomyces spp.*, (Tapia and Simoes2008), *B. Licheniformis* (Vigneshwaran2010). Some microorganism show optimum activity at the temperature of 60^0C.

Fig.1 Spore chain morphology under light microscope (isolate SH1)

Table.1 Characterization of isolate 2(SH1) (Williams *et al.*, 1983a)

Sr. No.	Characteristic		Result
1	**Morphological Characters**	Spore chain morphology (spirals)	+
2	**Pigmentation Characters**	Pigmentation on PYIA (Blackish brown)	+
3	**Carbon Utilization**	Glucose	+
		Sucrose	+
		Mannitol	+
		Xylose	+
		Arabinose	+
		Lactose	-
4	**Nitrogen utilization**	L-phenylalanine	
		L-Cysteine	-
		L-Histidine	+
		DL-Valine	+
5	**Enzyme activity**	Catalase	+
		Oxidase	+
		Lecithinase	+
		Lipolysis	+
		Protease	+
		Nitrate reductase	+
		Gelatinase	+
		Amylase	+
		Urease	+
		H2S production	-
6	**Growth Temperatures**	4^0C	-
		10^0C	-
		37^0C	+
		50^0C	-
7	**Growth in presence of inhibitory compounds**	Crystal violet (0.0001%)	-
		Phenol (0.1%)	+
		Sodium azide 0.001%	+
		Sodium azide 0.002%	-
		Sodium chloride 4%	+
		Sodium chloride 7%	-

* Where + = positive - = negative

Table.2 Amino acids from feather degradation using *Streptomyces coelicoflavus* in basal salt broth medium

Sr.No.	Amino acids	µg/ml of sample
1	Aspartic acid	0.47547
2	OH Proline	0.34055
3	Phosphoenolamine	0.1993
4	Serine	0.30448
5	Aspargine	0.27947
6	Taurine	0.76002
7	Threonine	0.4140
8	Proline	0.2004
9	Arginine	1.0853
10	1-Methyl histidine	0.59093
11	Tyrosine	0.29792
12	Valine	0.07203
13	Methionine	1.74876
14	Cystathionine	0.09125
15	Cysteine	3.35692
16	Leucine	2.02558
17	Phenylalanine	0.25544
18	Lysine	1.40148
	Total	**13.4986**

Table.3 Effect of metal on enzyme activity

Sr. No.	Divalent ions	Concentration	Relative activity (%)
	Control	-	100
1	Ca^{++}	5mM	108
2	Zn^{++}	5mM	101
3	Mg^{++}	5mM	108
4	Cu^{++}	5mM	101
5	Mn++	5mM	101

Fig.2 Spore chain morphology and arrangement under SEM

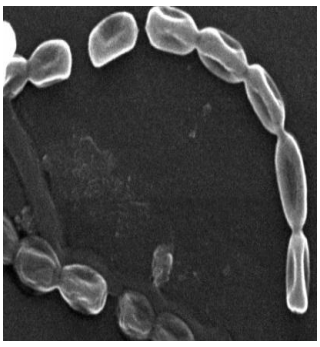
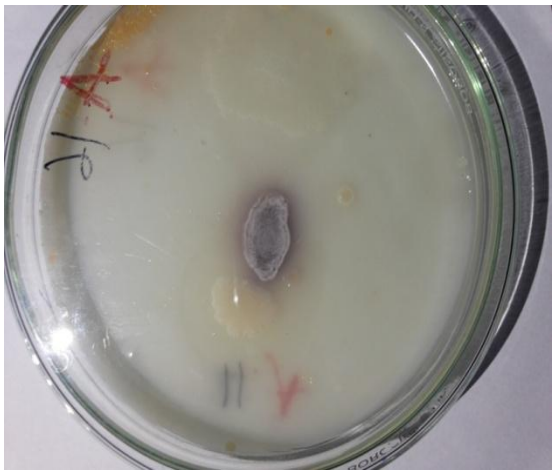


Table.4 Effect of Reducing agent and inhibitors on enzyme activity

Sr. No.	Reducing Agent	Concentration	Relative activity (%)
	Control	-	100
1	Dithiotreitol (DTT)	5mM	101
2	SDS	5mM	109
3	Sodium sulphide	5mM	111
4	DMSO	5mM	111
	Inhibitors		
5	1,10 Phenanthroline	5mM	106
6	EDTA	5mM	105

Fig.3 Clear zone around colony in Skimmed milk agar (isolate SH1).



Phylogenic tree

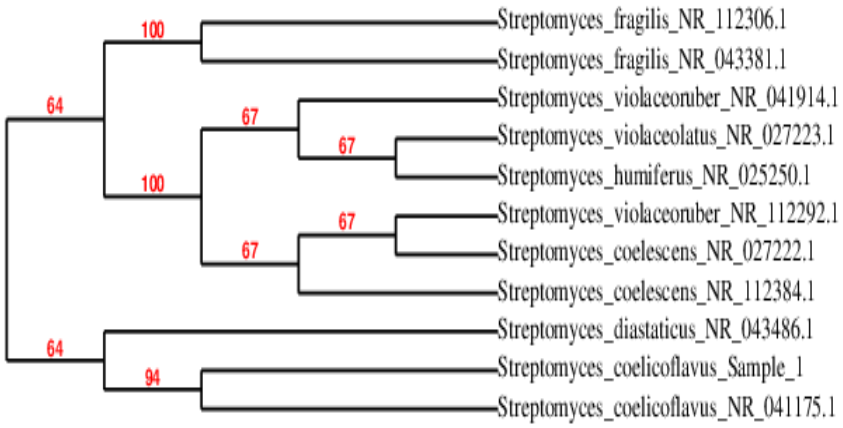


Fig.5 Chromatogram of HPLC (reverse phase) Amino acid analysis.

Amino acid analysed by HPLC

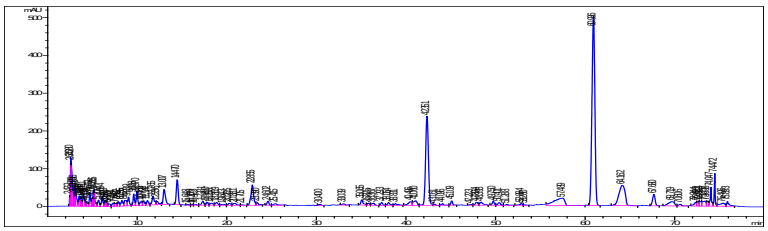


Fig.6 Effect of pH

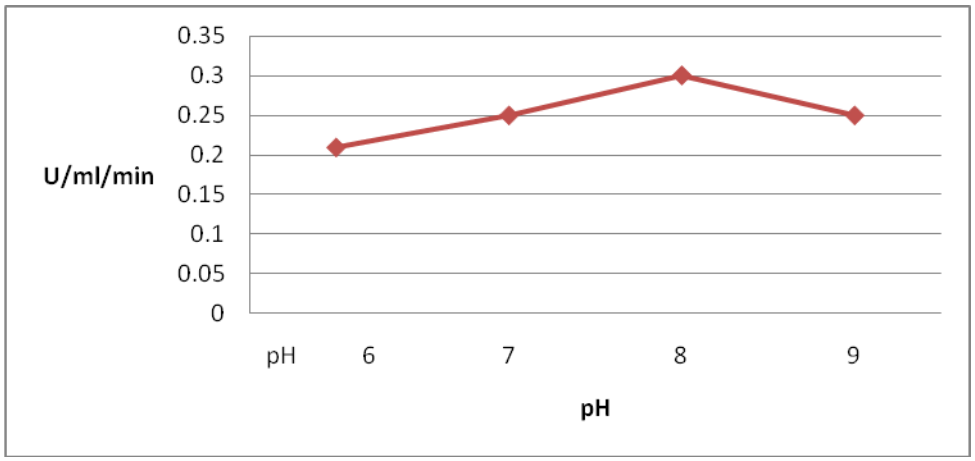
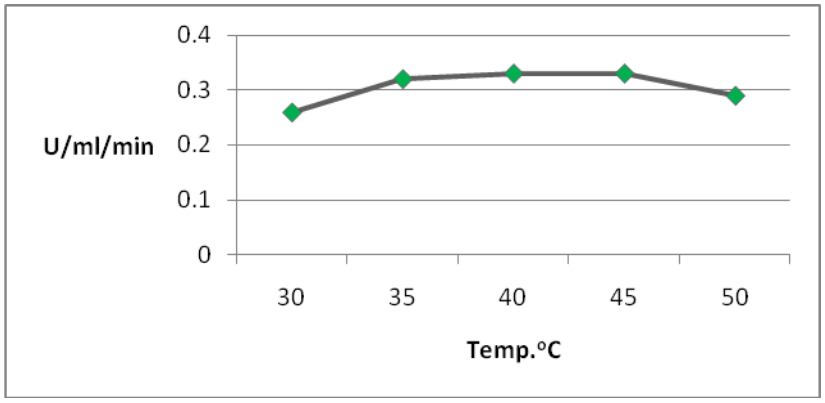


Fig.7 Effect Temperature



Effect of Divalent ion on enzyme activity

The result of enzyme activity of keratinase towards divalent ion (5mM) such as Ca^{++},Zn^{++}, Mg^{++}, Cu^{++}, Mn^{++}, were stimulatory. The effect of heavy metal ions such as Cu^{++} (Nam *et al.*, 2002), Mn^{++}, Mg^{++} on *B. subtilis* KD-N_2 strain was similarly reported by Bockel *et al.*, (1995).

Effect of Reducing agent and Inhibitors on keratinase enzyme

The keratinase activity was partially affected

by reducing agent Dithiotreitol (DTT) at a concentration 5mM. Sodium sulphide enhance the keratinase activity throughout the cleavage of disulphide bond reported by Letourneau in 1988. The SDS, 1,10Phenanthroline and EDTA (each 5mM) enhance effect on keratinase but less than sodium sulphide and DMSO. Similar results were observed by *Streptomyces pactum* DSMS 40536 (Bockle *et al.,* 1995).

In conclusion, according to these results, we conclude that the Keratinolytic proteainase of *Streptemces coelicoflavus LC072737* might be suitable for processing of keratinase containing waste like feather and hairs in leather industry under appropriate conditions. The crude keratinase was active at broad range of temperature (30^0C to 50^0C) and pH values (6 to 9) with optima at 40^0C and pH 8.

Acknowledgement

Researchers are thankful to the Principal of Shriman Bhausaheb Zadbuke Mahavidyalaya, Barshi Dist. Solapur for laboratory and library facilities to carry out this study and also thankful tothe Principal of Vishwasrao Naik Arts, Commerce and Baba Naik Science Mahavidyalaya, Shirala. Dist. Sanglifor Laboratory facilities to carry out this study.

References

Agraharisarita, Neerajwadhawa. 2010. Degradation of chicken feather a poultry waste product by Keratinolytic bacteria isolated from dumping site at ghazipur poultry processing plant. *Int. J. Poultry Sci.*, 9(5): 482-489.

Bockle, B., Galunsky, B., Muller, R. 1995. Characterization of Keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. *Appl. Environ. Microbial.,* 61: 3705-3710.

Bradford, M.M. 1976. Arapidand sensitive method for quantitation of microgram quantities of protein utilizing the principal of protein dye binding .*Anal. Biochem*., 72(12): 248-254, doi:10.1016/0003-2697(76)90557-3.

Bressollier, P., Letourneau, F., Urdaci, M., Verneuil, B. 1999. Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. *Appl. Environ. Microbiol.,* 65: 2570-2576.

Cai, C.G., Zheng, X.D. 2008. Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis. J. Zhejiang Univsci B.,* 9: 60-67.

Chitte, R.R., Nalawade, V.K., Dey, S. 1999. Keratinolytic activity from the broth of a feather-degrading thermophilic *Streptomyces thermoviolaceus* strain SD8. *Lett. Appl. Microbiol.,* 28: 131-136.

De Azeredo, L.A.I., De Lima, M.B., Coelho, R.R.R., Freire, D.M.G. 2006. Thermophilic protease production by *Streptomyces spp.* 594 in submerged and solid-state fermentations using feather meal. *J. Appl. Microbiol.,* 100(4): 641–647.

Ghosh, A., Chakrabarti, K., Chattopadhyay, D. 2008. Cloning of feather degrading minor extracellular protieases from *Bacillus cereus* DCUW: dissection of the structural domains. *Microbiol.*, 155: 2049-2057.

Jaouadi, B., Abdelmalek, B., Fodil, D., Ferradji, F.Z., Rekik, H., Zarai, N. 2010. Purification and characterization of a thermostablekeratinolytic serine alkaline proteinase from *Streptomyces spp.* Strain AB1 with high stability in organic solvents. *Bioresour. Technol*., 101: 8361-8369.

Kornillowicz, T. 1994. Method for determining Keratinolytic activity of saprophytic fungi, *Actamycologia,* 29(2): 169-178.

Kumar mukesh, D.J., Lavanya, S., Priya, P. 2012. Production of feather protein

concentrate from feathers by In *vitro* enzymatic treatment, its biochemical characterization and antioxidant nature. *Middle-East J. Scientific Res.*, 11(7): 881-886.

Lazim, H., Mankai, H., Slama, N., Barkallah, I., Limam, F. 2009. Production and optimization of thermophilic alkaline protease in solid state fermentation by *Streptomyces spp.*, CN902. *J. Microbiol. Biotechnol.*, 36(4): 531-537.

Onifade, A.A., Al-sane, N.A., Al-Musallam, A.A., AL-zardan, S. 1998. A review: potential for biotechnological application of keratin degrading microorganism and their enzyme for nutritional improvement of feathers and other keratins as livestock feed resources. *Bioresour. Technol.*, 66: 1-11.

Ponnuswamy Vijayraghavan, Lavanya, J., Samuel gnanaprakash Vincent. 2012. Biosynthesis and characterization of keratinilytic protease from *Actinobacterium spp.*, in solid state culture. *Inter. Jr. of Appl. Biol. Pharma. Tech.*, 3(2): 149- 158.

Pushpalata, S.K., Naik, G.R. 2010. Production and characterization of feather Degrading keratinase from *Bacillus Spp*. JB 99.*Indian J. Biotechnol.*, 9: 384-390.

Rayudu Krishna, Jayaraj. Y.M. 2014. Biodegradation of poultry feather by novel Keratinolytic bacteria isolates of North-East Karnataka, Ph.D. Thesis.

Saha Subhasish, Dhanasekaran, D. 2012. Exploration of Keratinolytic *Actinobacteria* for the bioconversion of poultry feather waste into poultry feed supplement, Ph.D. Thesis.

Setyorine, E., Takenaka, S., Murakami, S., Aoki, K. 2006. Purification and characterization of two novel halotolerant extracellular protease from *Bacillus subtilis* strain FP-133. *Bioscience. Biotechnol. Biochem.*, 70(2): 433-440.

Tapia, D.M.T., M.L.G. Simoes. 2008. Production and partial characterization of keratinase produced by a microorganism isolated from poultry processing plant wastewater. *Afr. J. Biotechnol*., 7: 296-300.

Turk, B. 2006. Targeting protease: Successes failures and feature prospects. *Nature Reviews Drug Discovery*, 5: 785-798.

Vigneshwaran, C., Shanmugam, S., Satishkumar, T. 2010. Screening and characterization of keratinase from *Bacillus licheniformis* isolated from namakkal poultry farm, 2(4).

Wawrzkiewicz, K., Wolski T., Lobarzewski, J. 1991. Screening the keratinolytic activity of dermatophytes in vitro. *Mycophthologia*, 114: 1-8.

Williams, S.T., Goodfellow, M., Alderson, G., Wellington, E.M.H., Sneath, P.H.A. and Sackin, M.J. 1983a. Numerical classification of *Streptomyces* and related genera. *J. General Microbiol.*, 129: 1743-1813.

Zardani, I.M., Faid, Malki, A. 2004. Feather wastes digestion by new isolated strains*Bacillusspp.*, In Morocco. *Afr. J. Biotechnol.*, 3: 67-70.

How to cite this article:

Jadhav, R.S., D.D. Karad and Kulakrni, S.W. 2016. Isolation, Identification and Characterization of Keratinolytic *Streptomyces coelicoflavus. Int.J.Curr.Microbiol.App.Sci.* 5(7): 153-163. doi: http://dx.doi.org/10.20546/ijcmas.2016.507.015