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Production of Keratinase by *Bacillus* sp. FK 28 Isolated in Thailand

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ABSTRACT

Screening for keratinase-producing bacteria and their keratinase production were investigated. Fifty two keratinase-producing bacterial strains were isolated from soils in Thailand on a semi-solid agar medium containing 5% feather powder as a substrate at 37°C. They produced keratinase in a range of 0.7-2.6 U/ml by shaking cultivation. The best keratinase producer was designated as FK 28 closely related to *Bacillus.* Keratinase was produced maximally in the medium containing 1% feather meal with an initial pH of 7.5 under cultivation conditions at 37°C and 150 rpm shaken.

Key words: *Bacillus* sp., feather, keratinase production

INTRODUCTION

Chicken feather mainly contains keratin, which is an insoluble protein with high stability and is indigestible by common proteases (Goddard and Michaelis, 1934; Papadopoulos, 1986). It is recognized as an abundant waste generated from poultry processing industry in Thailand (Anonymous, 1999). The feather can be hydrolysed by keratinase which is a proteolytic enzyme specific to keratins (Friedrich *et al*., 1999). The enzyme is a potential enzyme for removing hair and feather in the poultry industry (Takami *et al*., 1992), for nutritional upgrading of feather meal and conversion of feathers into a feed protein in feed industry (Williams *et al*., 1991), and for clearing obstructions in the sewage system during waste water treatment (Godfrey, 1996). This enzyme has been produced by fungi, including the species of *Aspergillus*, *Onygena*, *Absidia* and *Rhizomucor* (Friedrich *et al*., 1999), some species of dermatophytes, including *Trichophyton mentagrophytes*, *T. rubrum*, *T. gallinae*, *Microsporum canis* and *M. gypseum* (Wawrzkiewicz *et al.*,1991), a few actinomycetes such as *Streptomyces pactum, S. albus, S. fradiae* and *S. thermoviolaceus* (Bockle *et al.*, 1995; Chitte *et al*., 1999; Letourneau *et al*., 1998; Noval and Nickerson, 1959) and bacteria such as some *Bacillus* strains (Takami *et al*., 1992; Williams *et al.*, 1990) and the thermophilic *Fervidobacterium pennovorans* (Friedrich and Antranikian, 1996). In this paper we successfully isolated keratinase-producing bacteria from soils in Thailand and evaluated factors affecting bacterial keratinase production.

MATERIALS AND METHODS

Chemicals

Chicken feather and feather meal were supplied by a local poultry processing factory. The feather was ground by a ball mill to a feather powder. Standard proteins and tyrosine including important chemicals were purchased from Sigma Company.

Enrichment and isolation

Twenty soil samples were collected from

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areas in Bangkok and nearby provinces. To enrich keratinase-producing bacteria, five grams of soil were added to 250 ml-Erlenmeyer flasks containing 45 ml enrichment medium [1% (w/v) chicken feather in 0.1% (w/v) peptone]. The flasks were cultivated at the temperature of 30°C on a rotary shaker at 150 rpm for 30 days. During this enrichment, fresh medium was aseptically added every 10 days. The suspension was then plated onto a screening medium that contained (%, w/v): NH_4Cl 0.05, NaCl 0.05, K_2HPO_4 0.03, KH_2PO_4 0.04, $MgCl_2.6H_2O$ 0.024, yeast extract 0.01, agar 2, and feather powder 5, pH 7.5. The inoculated medium was incubated at 37° C for 3-5 days. A pure keratinase-producing culture was obtained by selecting a single colony surrounded by a clear zone. The cultures were kept on nutrient agar at 4°C until used.

Test for keratinase production

A full loop of each pure culture was added to 50 ml liquid medium containing (%, w/v): NH_4Cl 0.05, NaCl 0.05, K_2HPO_4 0.03, KH_2PO_4 0.04, $MgCl_2.6H_2O$ 0.024, yeast extract 0.01 and feather meal 1, pH 7.5 in a 250 ml-Erlenmeyer flask. The culture was grown on a rotary shaker at 150 rpm and incubated at 37°C for 24 h and used as an inoculum.

The enzyme production was done by inoculating 1 ml of each bacterial inoculum into 500 ml-Erlenmeyer flasks containing the same 100 ml liquid medium. The flasks were shaken at 150 rpm and incubated at 37°C for 3 days. The submerged cultures were carried out in duplicate. After 3 days of incubation, the broth was filtered through Whatman No.4 filter paper and the filtrate was collected to examine for keratinase activity and protein content.

Bacterial identification

Several bacterial strains producing high keratinase were identified. Bacterial cells were observed under light microscopy after Gram's and endospore stainings. Physiological and biochemical characteristics were studied followed Bergey's Manuals of Systematic Bacteriology (Kreig and Holt, 1984; Sneath *et al.*, 1986).

Factor affecting keratinase production

The best keratinase-producing strain was cultured under conditions described above for 5 days to examine the bacterial growth and its keratinase production. Factors affecting the keratinase production were investigated under the same conditions previously described. They were initial pH of the medium (pH 4.0-9.0), feather meal concentration (0.5-5.0%, w/v), cultured temperature (25-65°C) and shaking speed (100-250 rpm).

Keratinase assay

The keratinase activity was assayed by the modified method of Cheng *et al.* (1995). The mixture of 10 mg of feather powder suspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM $CaCl_2$ and 1 ml of culture filtrate was incubated at 45°C with shaking at 300 rpm for 30 min in a water bath shaker. This elevated temperature was used for the enzyme incubation to accelerate substrate hydrolysis. The enzyme reaction was terminated by adding 2 ml of trichloroacetic acid (TCA) solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid) into the reaction mixture. The mixture was then centrifuged at 3000 x g, 4°C for 30 min and the absorbance of the supernatant was spectrophotometrically measured at wavelength of 275 nm. The enzyme inactivated by TCA solution was used as a control. One unit (U) of keratinase activity was expressed as 1 μmol of tyrosine released per minute under the specific conditions.

Protein and bacterial growth determination

Protein content was analysed using the Lowry method with bovine serum albumin as standard protein (Lowry *et al*, 1951). Bacterial growth was determined by total plate count on nutrient agar.

RESULTS AND DISCUSSION

Isolation of keratinase-producing bacteria

A total of fifty two keratinase-producing bacterial strains was isolated from soil samples collected in Bangkok and nearby provinces (Phetchaburi, Nakhon Pratom and Suphanburi). They showed a clear zone around their colonies on the screening agar medium containing feather powder. The amount and particle size of feather powder in the medium affected the clarity of the clear zone around a colony. Five percent of feather powder (300 meshes) gave the most visible zone (data not shown). Soluble keratin was reported to employ as a substrate in an agar medium to select keratinase-producing fungi (Wawrzkiewicz *et al.*, 1991).

From preliminary study, an isolate produced the maximum keratinase after 3 days of cultivation (data not shown). All isolates yielded keratinase in a range of 0.7-2.6 U/ml as shown in Table 1. The strain FK 28 was able to produce the highest

Table 1 Keratinase production by bacterial isolates from soils in Bangkok and nearby provinces, Thailand.

Isolate	Keratinase (U/ml)	Isolate	Keratinase (U/ml)
B	2.12 ± 0.17	FK 31	1.23 ± 0.13
FK 1	1.56 ± 0.11	FK 32	1.25 ± 0.10
FK 2	1.62 ± 0.10	FK 33	2.38 ± 0.03
FK 3	1.32 ± 0.61	FK 36	1.23 ± 0.02
FK 4	0.73 ± 0.17	FK 39	1.62 ± 0.01
FK 5	1.35 ± 0.42	FK 40	1.91 ± 0.03
FK 6	1.05 ± 0.04	FK 41	1.35 ± 0.05
FK 7	0.99 ± 0.04	FK 42	2.21 ± 0.13
FK 8	1.45 ± 0.04	FK 43	1.72 ± 0.23
FK 9	1.10 ± 0.16	FK 44	1.18 ± 0.05
FK 10	1.79 ± 0.03	FK 45	0.98 ± 0.44
FK 11	1.80 ± 0.00	FK 46	2.05 ± 0.02
FK 13	0.89 ± 0.09	FK 47	1.10 ± 0.04
FK 14	1.94 ± 1.08	FK 48	1.09 ± 0.13
FK 15	1.12 ± 0.07	FK 49	1.58 ± 0.25
FK 16	1.34 ± 0.45	FK 50	1.75 ± 0.01
FK 17	1.68 ± 0.45	FK 51	1.34 ± 0.09
FK 18	0.94 ± 0.08	FK 52	1.32 ± 0.05
FK 19	1.24 ± 0.04	FK 53	1.95 ± 0.65
FK 20	0.77 ± 0.02	FK 54	2.12 ± 0.00
FK 21	1.60 ± 0.04	FK 55	1.79 ± 0.20
FK 23	1.56 ± 0.16	FK 56	2.00 ± 0.00
FK 24	0.98 ± 0.09	FK 57	1.80 ± 0.01
FK 26	2.23 ± 0.11	FK 58	1.47 ± 0.03
FK 27	1.06 ± 0.11	FK 62	1.63 ± 0.13
FK 28	2.60 ± 0.03	FK 65	1.87 ± 0.18
FK 29	1.61 ± 0.23		

keratinase activity of 2.6 U/ml with specific activity of 1.87 U/mg. It occurred singly or in chains and were straight rods (0.6 to 1.2 μm wide by 2.5 to 5.0 μm long in size), Gram positive and endospore-forming strain. It was aerobic, motile, and oxidase and catalase positive. From the morphological studies, stainings and biochemical examinations, the strain FK 28 was identified as the genus *Bacillus* (Table 2).

Bacillus species have been known to produce a number of hydrolytic enzymes including keratinase, which is able to degrade feathers, wool and hair. Williams *et al.* (1990) found a straight rod shaped, aerobic and endospore-forming bacterium appeared in singly and in chains, catalase positive and Gram variable, classified as *Bacillus licheniformis* PWD-1. This bacterium was isolated from a high temperature poultry waste digestor and was able to degrade feather keratin when using feathers as a primary source of carbon and energy. Atalo and Gashe (1993) described a thermophilic *Bacillus* species that could produce protease to degrade various fibrous protein such as feather, hair, sheep skin or horn. In addition, Takami *et al.* (1989, 1992) reported a rod-shaped and spore-forming bacterium, identified as *Bacillus* sp. No. AH-101, that produced a thermostable alkaline protease to degrade human hair. Friedrich and Antranikian (1996) described that a keratin-degrading strain of a thermophilic anaerobic bacteria, rod-shaped with an outer sheath-like structure of 2.0-20 μm long, occurred singly or in pairs, Gram negative and no endospore-forming. Lal *et al*. (1999) reported *Bacillus* spp. were able to

Table 2 Some characteristics of the strain FK 28 and a species of *Bacillus*.

Characteristics	FK 28	*Bacillus* sp.
Rod-shaped	+	+
Diameter over 2.5 μm	-	-
Gram reaction	+ or variable	+
Endospore produced	+	+
Motility	+	+
Anaerobic growth	-	-
Catalase	+	+
Oxidase	+	D
Voges-Proskauer reaction	-	-
Indole production	-	-
Nitrate reduction	-	D
Growth in 5% NaCl	+	D
7% NaCl	+	D
10% NaCl	-	D
Starch hydrolysis	-	-
Casein hydrolysis	+	+
Temperature for growth (°C)	RT to 45	RT to 65
Citrate utilization	+	D
Urea hydrolysis	+	D

Symbols: + positive; - negative; D substantial proportion of species differ;
RT room temperature

degrade keratin substrates from human hair, human nails, cow horn and cow hooves *in vitro*.

Factors affecting keratinase production

Bacterial growth and keratinase production by *Bacillus* sp. FK 28 during cultivation were illustrated in Figure 1. The keratinase activity was maximal during late exponential growth after 48 h cultivation (2.6 U/ml) and rapidly decreased during the stationary phase. Similar result was found in protease production by other *Bacillus* strains (Atalo and Gashe, 1993; Cheng *et al*., 1995).

The amount of keratinase production depended on substrate concentrations and cultivation conditions. Feather meal concentration, initial pH, shaking speed and culture temperature were affected keratinase production (Figures 2-5). The highest keratinase production was obtained at 1% feather meal (Figure 2). Cheng *et al*. (1995) also reported that 1% feather powder gave the highest keratinase activity for *B. licheniformis* PWD-1. Keratinase produced at a level of 0.5% feather meal was ten times less than that produced at 1% feather meal. This is because the amount of substrate supplied for growth and enzyme production was insufficient, while 2.5% and 5% feather meal showed substrate repression on keratinase production. High feather meal concentration also increased medium viscosity which possibly resulting in oxygen limitation for bacterial growth. Similar finding was found with high feather concentration (2%) but lower protease production by *Streptomyces thermonitrificans* (Mohamedin, 1998).

Maximal keratinase production was obtained when an initial pH of medium was 7.5 (Figure 3). Poor growth was observed when initial pHs were at 4.0, 6.0 and 9.0. Altalo and Gashe (1993) found the optimum pH of keratinase production by *Bacillus* species P-001A at pH 9.5. Cheng *et al* (1995) also reported the highest keratinase production by *B. licheniformis* PWD-1 was at an initial pH of 8.7.

The shaking speed at 150 rpm yielded maximum keratinase production (Figure 4). Generally, increased shaking speed provided high oxygen transfer rate supporting cell growth. However, high shaking speed (200-250 rpm) gave good bacterial growth but low keratinase production possibly because of too high dissolved oxygen and too much shear stress repressed keratinase synthesis and excretion. Perlman (1969) reported that vitamin

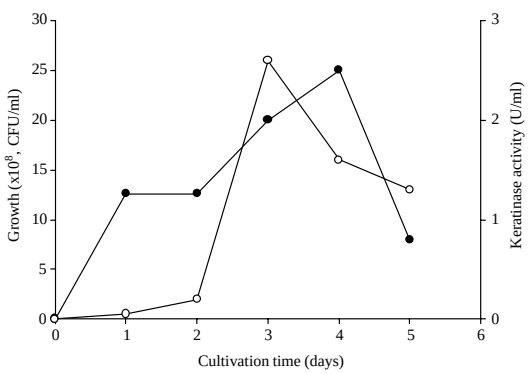

Figure 1 Growth (•) and keratinase (o) production by *Bacillus* sp. FK 28 cultivated in liquid medium containing 1% feather meal as a substrate at pH 7.5, 37°C and 150 rpm shaken.

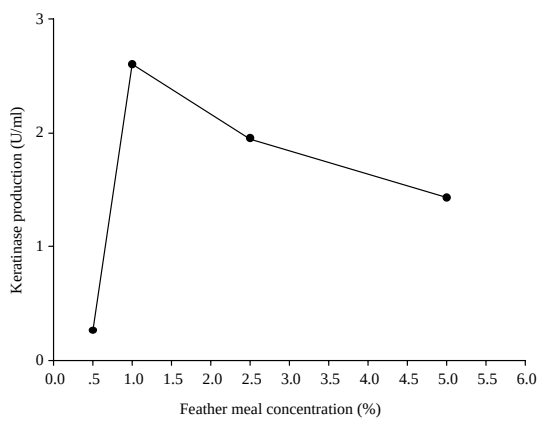

Figure 2 Effect of feather meal concentration (%) on keratinase production by *Bacillus* sp. FK 28 at pH 7.5, 37°C and 150 rpm shaken.

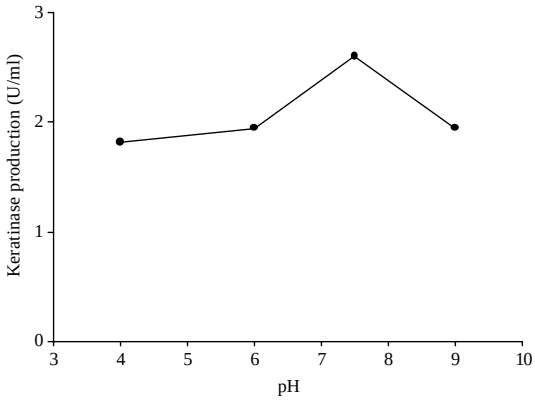

Figure 3 Effect of initial pH on keratinase production by *Bacillus* sp. FK 28 in 1% feather meal at 37°C and 150 rpm shaken.

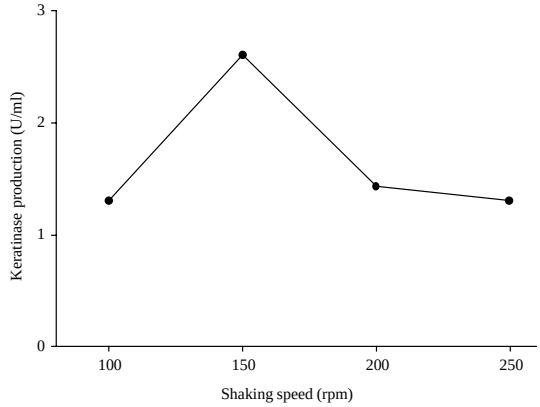

Figure 4 Effect of shaking speed on keratinase production by *Bacillus* sp. FK 28 in 1% feather meal at pH 7.5 and 37°C.

B_{12}-producing enzyme was inhibited by high aeration. At low shaking speed (100 rpm), bacterial cells and substrate were not well mixed with heterogeneous distribution and lower oxygen dissolved resulting in low keratinase production.

The highest bacterial growth and keratinase production were found at the incubation temperature of 37°C (Figure 5). No keratinase production was observed at 50°C and 65°C because of an absence of bacterial cell growth at such high temperatures. Poor growth and 50% less keratinase production were found at 25°C. Compared with *B. licheniformis* PWD-1, the maximum growth was reported at 50° C (Cheng *et al*., 1995; William *et al*., 1990), while the maximum enzyme production was obtained at 45°C (Cheng *et al*., 1995).

In conclusion, a bacterial strain designated as FK 28, isolated from soils in Thailand, is closely related to *Bacillus*. It was able to produce keratinase and could be applied for feather degradation into feather protein hydrolysate used in feed industry. Its keratinase production was maximal in a medium containing 1% feather meal as a substrate, pH 7.5, and under cultivation conditions at the temperature of 37°C and the shaking speed of 150 rpm. Identification of bacterial isolates by a rapid biochemical test kit and 16s rRNA analysis is under investigation.

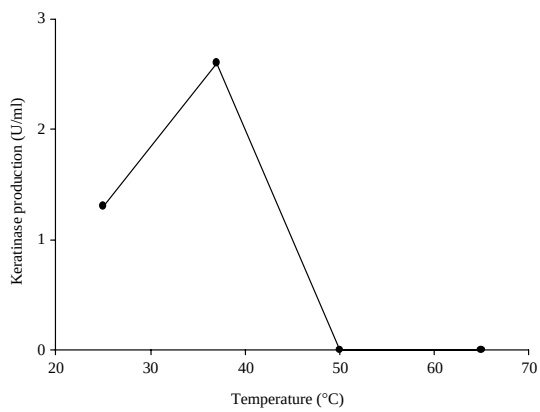

Figure 5 Effect of cultivation temperature on keratinase production by *Bacillus* sp. FK 28 in 1% feather meal at pH 7.5 and 150 rpm shaken.

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