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Fermentation production of keratinase from *Bacillus licheniformis* PWD-1 and a recombinant *B. subtilis* FDB-29

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Fermentation scale-up was studied for the production of keratinase by *Bacillus licheniformis* PWD-1, the parent strain, and *B. subtilis* FDB-29, a recombinant strain. In both strains, keratinase was induced by proteinaceous media, and repressed by carbohydrates. A seed culture of *B. licheniformis* PWD-1 at early age, 6–10 h, is crucial to keratinase production during fermentation, but *B. subtilis* FDB-29 is insensitive to the seed culture age. During the batch fermentation by both strains, the pH changed from 7.0 to 8.5 while the keratinase activity and productivity stayed at high levels. Control of pH, therefore, is not necessary. The temperature for maximum keratinase production is 37°C for both strains, though *B. licheniformis* is thermophilic and grows best at 50°C. Optimal levels of dissolved oxygen are 10% and 20% for *B. licheniformis* and *B. subtilis* respectively. A scale-up procedure using constant temperature at 37°C was adopted for *B. subtilis*. On the other hand, a temperature-shift procedure by which an 8-h fermentation at 50°C for growth followed by a shift to 37°C for enzyme production was used for *B. licheniformis* to shorten the fermentation time and increase enzyme productivity. Production of keratinase by *B. licheniformis* increased by ten-fold following this new procedure. After respective optimization of fermentation conditions, keratinase production by *B. licheniformis* PWD-1 is approximately 40% higher than that by *B. subtilis* FDB-29.

Keywords: keratinase production; fermentation; *Bacillus*; recombinant strain

Introduction

A feather-degrading *Bacillus licheniformis* PWD-1 was discovered and isolated in this laboratory [33]. The enzyme keratinase produced by the strain was purified and characterized as a serine protease [15]. In addition to promoting the hydrolysis of feather keratin, it hydrolyzes a broad range of proteins. Preliminary nutritional studies indicated that supplementation of keratinase in feed can improve the digestibility and nutritional value of feather meal in chickens [12]. The gene (*kerA*) which encodes keratinase in the strain was isolated and sequenced [16]. Sequence analysis indicated that keratinase belongs to the subtilisin superfamily [28]. A recombinant strain, *B. subtilis* FDB-29, which carries the P43 promoter and over-expresses *kerA* was subsequently developed by genetic transformation [17].

Fermentation conditions, scale-up procedures, and gene regulation are all involved in successful production of an enzyme by fermentation. Optimization of parameters including medium composition, pH, dissolved oxygen, and temperature is important in developing the fermentation process. Due to a large divergence in physiological patterns found in *Bacillus* species, cultivation conditions promoting enzyme production vary widely. Previous studies have reported that the formation of desired bioproducts is frequently not consistent with the cell growth. In some cases, the production of desired products is inversely related to cell growth [14,19,27].

In order to have sufficient quantities of keratinase for application research in feed and other areas, a scale-up fermentation process was studied for the production of this enzyme in a larger quantity than that obtained at the laboratory-scale. A fermentation facility consisting of a 15-L bench-top fermentor, a 150-L industrial fermentor and other accessories were used. Fermentation parameters for growth and enzyme production were optimized in the tightly controlled bench-top fermentor. One-hundred liter cultures in the industrial fermentor were used for production and harvest of keratinase. Although *B. subtilis* FDB-29 was developed to over-express keratinase, the work was done in small flasks [17]. No further evidence was reported to indicate that *B. subtilis* FDB-29 is a preferred strain for scale-up in fermentors to produce keratinase. Optimal scale-up procedures in batch fermentation for both strains were individually developed. Results of these studies are reported in this communication.

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Materials and methods

Bacterial strains.

B. licheniformis PWD-1 (ATCC 53757) was originally isolated in this laboratory from a poultry waste digestor [33]. The keratinase gene, *kerA*, was isolated from strain PWD-1 [16]. Transformation of *B. subtilis* DB104, a protease-deficient strain, with plasmid pLB29, which harbors *kerA*, promoter P43, and kanamycin resistance gene, resulted in the recombinant *B. subtilis* FDB-29 [17]. Stock cultures of *B. licheniformis* PWD-1 in nutrient broth (Difco Laboratories, Detroit, MI, USA) and *B. subtilis* FDB-29 in Luria-Bertani broth (LB) containing 20 mg L^{-1} kanamycin were stored at −70°C in 50% glycerol. *B. licheniformis* PWD-1 grows the best at 50°C, *B. subtilis* FDB-29 grows best at 37°C.

Fermentation media and growth conditions

Fermentation media were prepared by adding various amounts of organic substrates, typically 0.5% ground feathers, to the phosphate basal solution with initial pH at 7.0 (0.5 g NaCl, 0.7 g KH_2PO_4, 1.4 g K_2HPO_4 and 0.1 g $MgSO_4 \cdot 7H_2O$ per liter of water). When carbohydrates were used as substrates, NH_4Cl (0.1%) was added as the source of nitrogen. Test tube and flask culture media were sterilized at 121°C for 30 min by autoclaving. Bioflow III fermentor (10-L working volume) with medium was sterilized at 121°C for 70 min. Media in an IF150 fermentor (100-L working volume) was sterilized *in situ* at 121°C for 45 min. To avoid the loss of the *kerA*-carrying plasmid in strain FDB-29, kanamycin (20 mg L^{-1}) was always added in the media for the recombinant strain.

A seed culture was prepared by streaking a nutrient agar plate with PWD-1 cells from a stock culture. A single colony from the plate was transferred to 10 ml of seed culture medium, incubated at 50°C until the cell density reached mid-log phase (5×10^8 cells ml^{-1}), and inoculated to the next stage. Inoculum of strain FDB-29 was prepared the same way, except that the stock culture was streaked on tryptose blood agar base (TBAB) (Difco Laboratories) plates containing 20 mg L^{-1} kanamycin. The seed culture was grown at 37°C.

Fermentation facility and operation

Optimization of fermentation was carried out with a 15-L fermentor (Bioflow III) equipped with an interface controller capable of monitoring and controlling the fermentation parameters, including pH, temperature, dissolved oxygen (DO), agitation, and foaming. All data were displayed or printed using a dedicated PC with the AFS-BioCommand bioprocessing software. Large-scale production of keratinase was accomplished with a 150-L fermentor (IF-150) with a ML4100 control system (New Brunswick Scientific, Edison, NJ, USA).

In the Bioflow III, the agitation range was limited to 300 rpm with a 3 L min^{-1} air flow rate to maintain the specified DO level from 5% to 40%. In the IF-150, air flow rate ranged from 15 to 30 L min^{-1} in order to limit agitation speed to 200 rpm when the DO was maintained at 10% saturation.

Fermentation pH was controlled in the range from 6.5 to 8.5 by adding 1 N NaOH or 1 N HCl using a pH controller. Temperature from 37 to 50°C and DO from 5% to 40% were tested during fermentations. The dissolved oxygen was detected by a DO probe (Model Ingold, New Brunswick Scientific) connected to a DO analyzer. Foaming was controlled by addition of 1% Sigma (St Louis, MO, USA) Antifoam 289 solution as required.

Keratinase was harvested by continuous centrifugation (Model CEPA-Z81, Lahr, Germany) followed by concentration of the supernatant using a Pellicon filtration system (Millipore, Bedford, MA, USA) with a 10 kDa MW cut-off membrane and then lyophilized.

Measurement of growth

Total biomass, protein concentration and keratinase activity were determined via off-line assay of culture samples. Cell numbers were estimated by direct cell count in a counting chamber under a phase-contrast microscope. Optical density at 660 nm could be monitored for culture growth in nutrient broth but not in the feather suspension medium. The dry cell weight was measured by centrifuging 20 ml of culture, removing the supernatant, resuspending the cells in an equal volume of distilled water, and drying them at 105°C for 24 h. Dry weights were given as the mean of samples in triplicate. Results from all three methods correlated with one another.

Isolation and determination of messenger RNA of kerA

A method for the isolation of RNA from *B. licheniformis* PWD-1 cells grown in feather medium at 37°C or 50°C was developed as modified from a common procedure for *E. coli* [26]. The cell pellet from 10 ml medium was collected by centrifugation at $7000 \times g$ for 10 min at 4°C, added with 1 μl (40 U) RNAsin RNase inhibitor (Promega, Madison, WI, USA) and 1.0 ml TRIzol reagent (Gibco BRL, Rockville, MD, USA) and stored at –70°C. The cell pellet was then thawed on ice, and added to 0.7 g 106-μm glass beads (Sigma Chemical Co) in a 2.0-ml screw-cap vial. Samples were homogenized in a MiniBeadbeater-8 cell disrupter (Biospec Products, Bartlesville, OK, USA) for six 1-min cycles, chilling the samples on ice between cycles. To separate the phases, 0.2 ml of chloroform was added to each sample, which was homogenized for 15 s, and centrifuged at $10\,000 \times g$ for 15 min at 4°C. The clear upper layer was transferred to a fresh tube, and the RNA was precipitated by adding 0.5 ml isopropanol, incubating the mixture at room temperature for 10 min, and centrifuging it at $10\,000 \times g$ for 5 min at 4°C. After washing the RNA pellet with 75% ethanol, it was air dried and then digested with 5 units RQ1 DNase (Promega, catalog No. PR-M6101).

Total RNA, 2.5 μg for each sample, was denatured by boiling the solution for 10 min. It was incubated on ice for 3 min, and applied to Super Strength membrane using a MiniFold dot blot manifold, model SRC-96 (Schleicher and Schuell, Keene, NH, USA). The membrane was baked at 120°C for 30 min.

Digoxigenin-labeled probes were amplified from *B. licheniformis* genomic DNA by PCR using the PCR DIG Labeling mix (Boehringer-Mannheim, Mannheim, Germany), as recommended by the manufacturer. Primers used to amplify a digoxigenin-labeled 1.2-kb fragment containing the *kerA* coding region [16] were KERA3 (5′-ATTTAAATTATTCTGAATAAAGAGG-3′) and KERA4 (5′-CACTAGCTTTTTCTATATGCTATTTG-3′). Primers used to amplify digoxigenin-labeled 0.7-kb fragment containing the 16S rRNA gene were 704F (5′-GTAGCGGTGAAATGCGTAGA-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). RNA dot blots were hybridized to these probes using DIG Easy Hyb hybridization buffer (Boehringer-Mannheim), and detected with Anti-DIG-AP antibody (Boehringer Mannheim, No. 1093274) and CSPD chemiluminescent substrate (Boehringer Mannheim). All RNA dot blot hybridization and detection procedures were adopted from the DIG System User Guide for Filter Hybridization (Boehringer-Mannheim).

Keratinase assay and protein determination

The total protein concentration in samples was measured using the Bio-Rad Microassay Procedure [2]. Keratinase activity was measured by azokeratin hydrolysis as described previously [15].

Results

Substrate effect on keratinase production

The effects of various substrates on keratinase expression were investigated. Carbohydrates, including glucose, as sole carbon sources repressed *kerA* expression, while proteinaceous substrates allowed expression of keratinase in both strains. Casamino acids repressed keratinase in *B. licheniformis* but not in *B. subtilis* (Table 1). In media containing 0.5–1.5% ground feathers (Figure 1), *B. licheniformis* grew more as the substrate increased, but produced keratinase inversely to cell growth. In contrast, *B. subtilis* showed similar growth characteristics, but enzyme production generally reflected cell growth and plateaued at 1.0%. For keratinase production, 0.5% feather medium for *B. licheniformis* and 1.0% feather medium for *B. subtilis* were used.

Inoculum age

The effects of inocula at different ages on keratinase production are illustrated in Figure 2. Each fermentation was started with an identical seed culture from feather medium, except that inocula were at different ages. The age of seed culture did not have any effect on growth of either strain in reaching maximum cell density, 10^9 ml^{-1}, and had no effect on keratinase production by *B. subtilis*, but production of keratinase by *B. subtilis* decreased with older inocula. Seed cultures at 6–10 h caused the fermentation to produce twice as much keratinase as those at 24 and 42 h.

Table 1 Keratinase production in both cultures

Substrate	Keratinase activity (U ml^{-1})		
	PWD-1		FDB29
	0.5% FM 37°C	1.0% FM 50°C	1.0% FM 37°C
Ground feather	450 ± 28	200 ± 15	310 ± 20
Commercial feather meal	510 ± 22	240 ± 10	303 ± 16
Soybean flour	475 ± 18	240 ± 15	315 ± 8
Tryptone	300 ± 22	170 ± 10	190 ± 7
Yeast extract	310 ± 23	180 ± 20	170 ± 15
Casamino acids	55 ± 12	40 ± 10	176 ± 9
Nutrient broth	150 ± 22	120 ± 11	172 ± 9
Glucose	16 ± 5	12 ± 3	16 ± 2
Sucrose	33 ± 8	40 ± 5	11 ± 3
Starch	35 ± 6	21 ± 4	33 ± 6
Lactose	15 ± 6	16 ± 3	17 ± 2
Molasses	14 ± 8	20 ± 5	26 ± 3

[a]Cultures were grown, 50 ml medium in a 250-ml flask, in a shaker at 200 rpm.

pH during fermentation

In comparing the effect of pH on keratinase production, two trends were observed. *B. licheniformis* exhibited best keratinase activity under neutral conditions (pH 7.0–7.5) or even better without pH control (7.0 initial and 8.5 final), whereas *B. subtilis* tended to do best in a weakly alkaline environment (pH 8.0–8.5) and about the same without pH control (Table 2).

Keratinase productivity is defined as the peak enzyme activity divided by the number of hours to reach the peak. For *B. licheniformis*, growth without controlled pH gave comparable enzyme productivity to pH 7.0 and 7.5, although peak enzyme activity was relatively higher without pH control. In contrast, in *B. subtilis*, growth under a controlled pH from 7.5 to 8.5 gave no apparent advantage over no pH control, with regards to either enzyme productivity or peak activity (Table 2). Thus, uncontrolled pH is to be preferred for optimum production of keratinase in both organisms. Growth and enzyme production under uncontrolled pH are illustrated in Figure 3.

DO in fermentation

The effect of DO on cell growth, growth rate (cell density/time), keratinase activity, and productivity (activity/time) is presented in Figure 4. Growth rate and enzyme productivity by both organisms were reduced by limited DO at 5%. Cell growth rate of *B. licheniformis* was greatly reduced at a DO level above 20%, but the growth rate of *B. subtilis* was not affected. DO levels higher than 20% apparently decreased enzyme production in both strains. Optimal DO tensions for the production of keratinase by both strains were 10% and 20% respectively (Figure 4).

Temperature effects

Optimal cell growth for PWD-1 took place at 50°C with a cell doubling time of 4–5 h (data not shown), which was consistent with previous results [33]. Higher keratinase activity, however, was observed at 37°C instead of 50°C (Figure 5), while the cell doubling time at 37°C was twice as long as at 50°C (data not shown). The keratinase activity increased about 45% in 0.5% feather medium when the temperature was decreased from 50°C to 37°C. From this experiment, it was concluded that higher production of keratinase occurred at 37°C, although the cell-growth optimum is at 50°C. This observation was further supported by the result of Northern blot analysis (Figure 6). The transcription level of mRNA of *kerA* isolated from cells growing at 37°C is much higher than that from cells at 50°C.

Cells of *B. subtilis* stopped growing when temperatures were over 55°C. Maximum keratinase activity occurred at 37°C and maximum growth was observed at 42°C. Maximum enzyme productivity occurred at 42°C, because the fermentation time can be shortened (Figure 5).

Fermentation scale-up

Several procedures were designed to raise enzyme productivity. When nutrient broth was used as a substitute for feather medium for *B. licheniformis* in flasks, it shortened culture time for seed cultures from 8 h to 4 h. In a 10-L fermentor, feather medium was used in order to reduce the

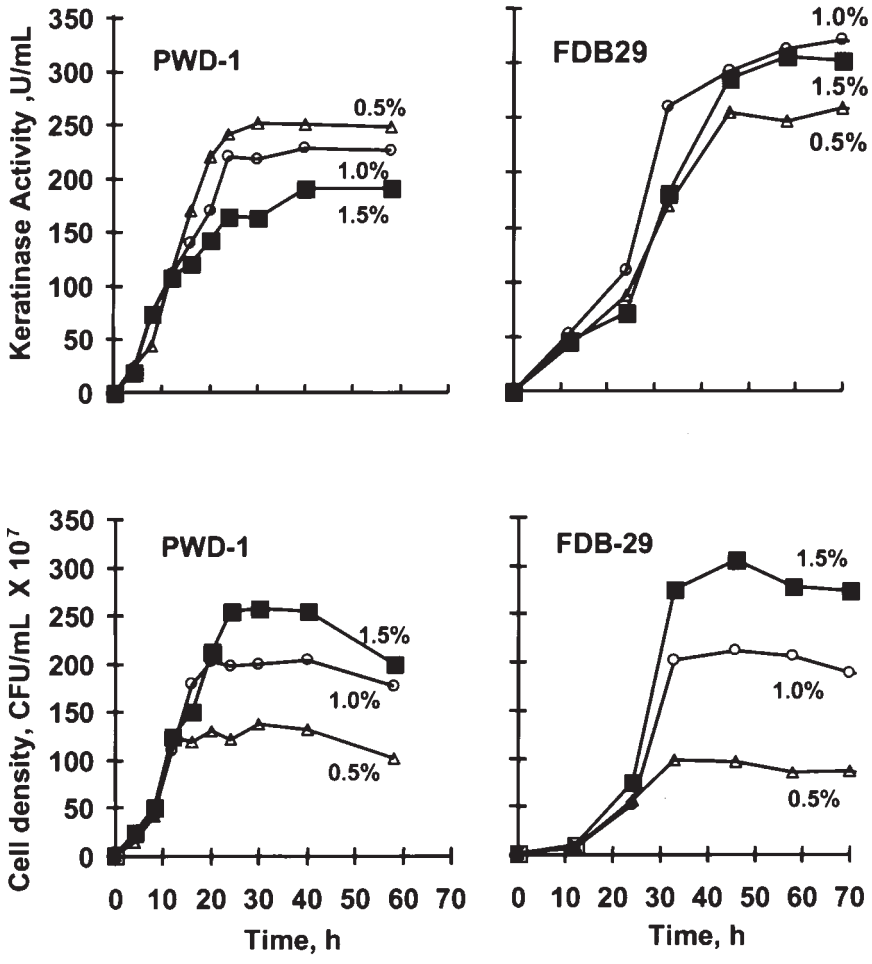

Figure 1 Effects of concentration of ground feathers on keratinase production and cell growth. Experiments were done in flask cultures, incubated at 50°C for *B. licheniformis* and 37°C for *B. subtilis*.

cost and possibility of contamination. After 8 h at 50°C, it was inoculated in an 100-L feather medium in the IF-150 fermentor for keratinase production.

Since growth of *B. licheniformis* at 50°C is faster than at 37°C, processes were tested to determine if keratinase production could be improved (Figure 7). When growth conditions was held at 50°C and pH 7.5 for 8 h followed by continued fermentation at 37°C without pH control (curve b), the peak of keratinase activity was shifted approximately 10 h earlier, compared with the fermentation held at 37°C (curve a). However, keratinase production was restricted when 50°C was maintained for 12 h (curve c), which corresponds to peak enzyme production during fermentation at constant 50°C (curve d). Although a decrease in temperature after 12 h did not increase enzyme production, it helped to stabilize enzyme activity (curve c) *vs* constant 50°C fermentation (curve d). The appropriate temperature shift in the fermentation process not only enhanced enzyme productivity, but maintained a high activity for a longer time (curve b).

Scale-up procedures for *B. subtilis* were also tested. The age of inoculum had little effect on production of keratinase. LB broth or feather media containing 0.1% yeast extract shortened the seed culture time, increasing the overall enzyme productivity. Feather media were used for 10-L and 100-L fermentations. Because both cell growth and keratinase production were optimal at 37°C, the scale-up fermentation was held at 37°C. The performances of both strains at different sizes of fermentation are summarized and compared in Table 3.

Keratinase production cost

Three low-cost common substrates: ground feathers, commercial feather meal, and soy flour, were used to compare their relative costs for the production of keratinase. Cost factors of commercial feather meal and soy flour were derived from current market price, whereas the price of ground feathers was assumed to be equal to that of commercial feather meal. The relative cost and enzyme yields comparing three different substrates are summarized in Table 4. The most cost-effective production was achieved by using ground feathers or commercial feather meal by *B. licheniformis*. For *B. subtilis*, soy flour supported a higher keratinase activity than ground feathers or commercial feather meal.

The highest keratinase yield of *B. licheniformis* was 54.7 mg keratinase g^{-1} biomass when fermented in commercial feather meal, and for *B. subtilis*, 16.2 mg keratinase g^{-1} biomass when fermented in soy flour. Production costs based on ground feathers or commercial feather meal as

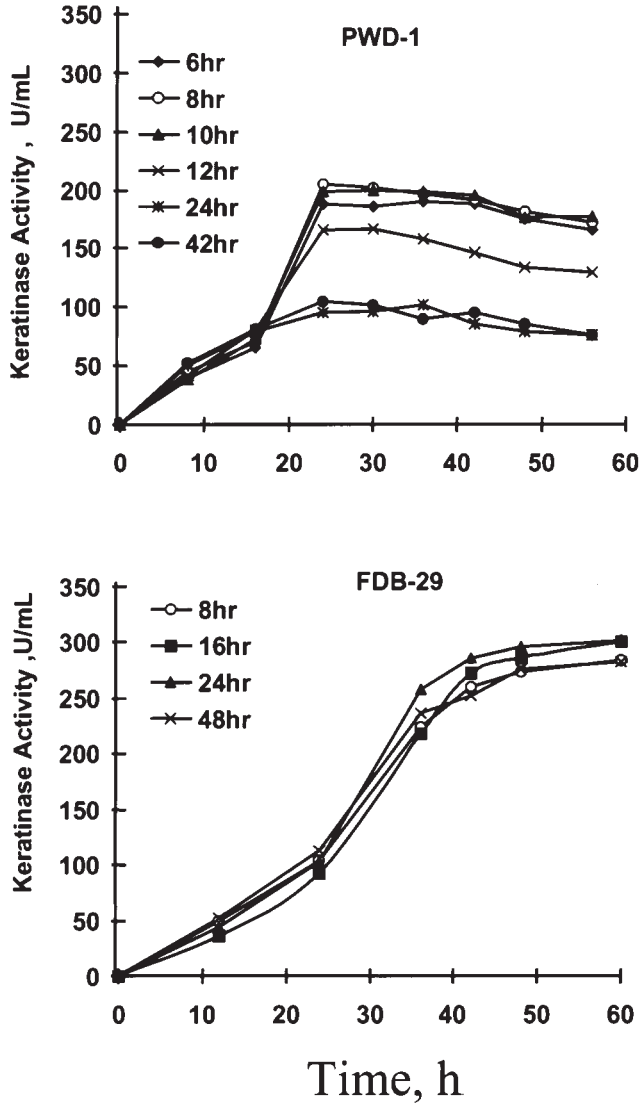

Figure 2 Keratinase activity in batch cultures of *B. licheniformis* and *B. subtilis* with different inoculum age. *B. licheniformis* was grown at 50°C and *B. subtilis* was grown at 37°C, in 1% feather medium.

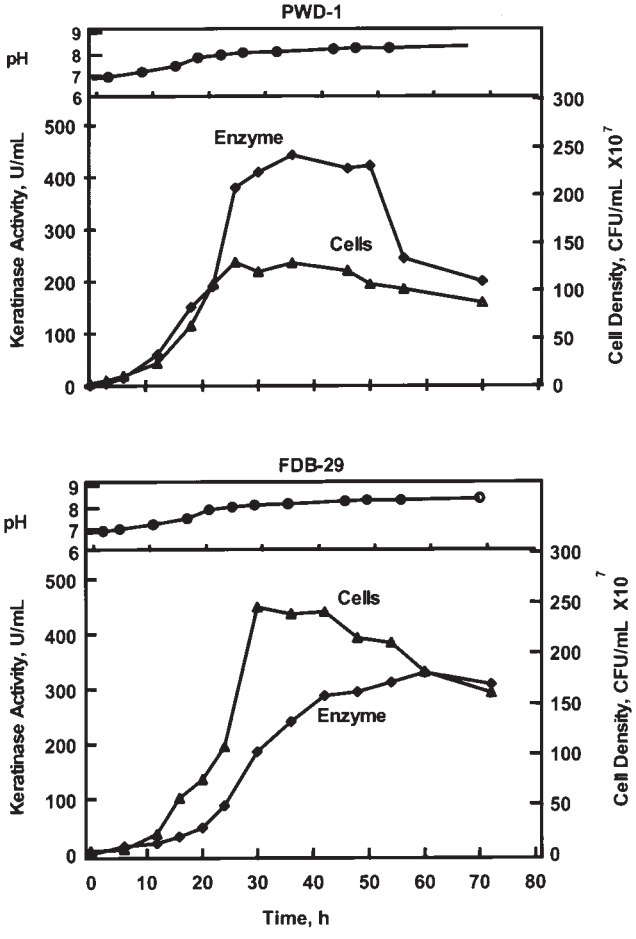

Figure 3 Relationship of cell growth, keratinase yield, and pH variation during fermentation by *B. licheniformis* and *B. subtilis*. *B. licheniformis* was grown at 37°C, 0.5% FM, and 10% DO, and *B. subtilis* was grown at 37°C, 1.0% FM, and 20% DO.

Table 2 Keratinase activity and productivity at different pH

pH	*B. licheniformis*[a]		*B. subtilis*[a]	
	Activity[b]	Productivity[b]	Activity	Productivity
6.5	145	4.0	142	2.4
7.0	163	13.6	168	2.9
7.5	160	13.3	280	6.7
8.0	120	7.5	312	6.8
8.5	20	1.3	300	5.0
Uncontrolled[c]	220	13.8	311	5.2

[a]*B. licheniformis* was grown in 0.5% FM at 50°C; *B. subtilis* in 1.0% FM at 37°C.
[b]Activity = U ml^{-1}; productivity = Activity h^{-1} to reach peak activity.
[c]pH changed from 7.0 to 8.5 during fermentation.

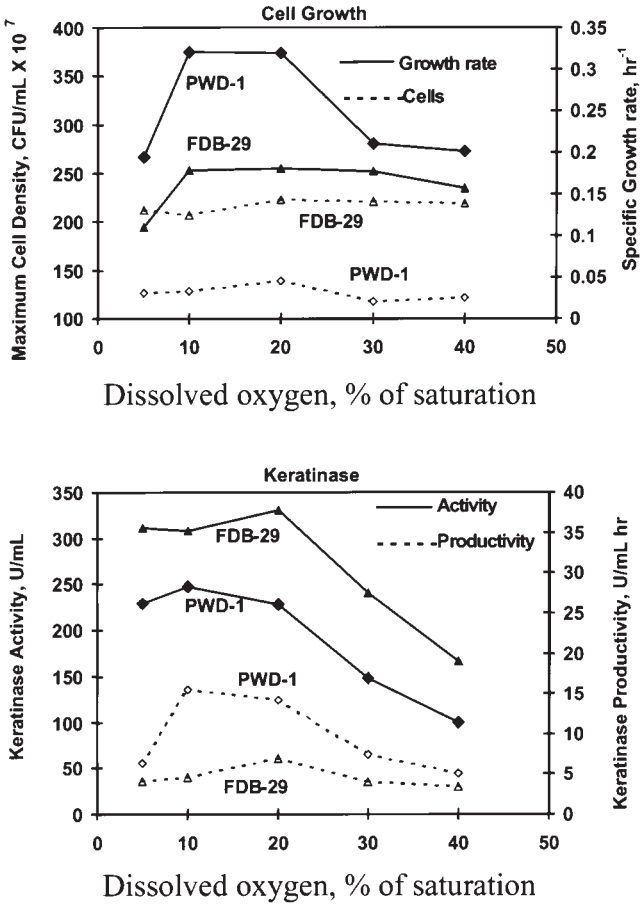

Figure 4 Effect of dissolved oxygen on cell growth and keratinase productivity in 15-L fermentor. *B. licheniformis* was conducted in 0.5% FM at 50°C, and uncontrolled pH; *B. subtilis* was conducted in 1.0% FM at 37°C, and uncontrolled pH.

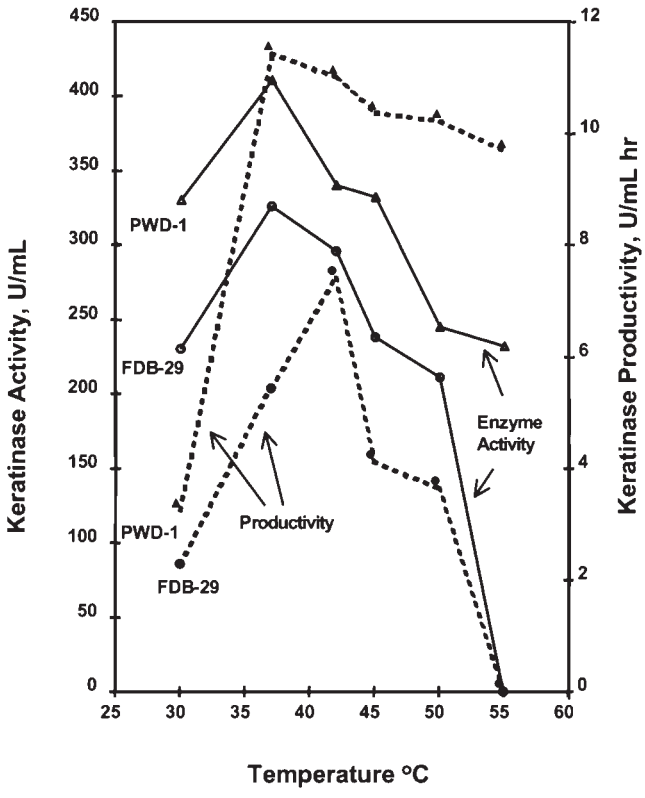

Figure 5 Keratinase activity and productivity during the fermentation of *B. licheniformis* and *B. subtilis* at different temperatures. All experiments were conducted in flask cultures. *B. licheniformis* was grown in 0.5% FM; *B. subtilis* in 1.0% FM.

the substrate are significantly lower than that of soy flour for both organisms. Ground feathers, without pressure cooking as commercial feather meal is processed, was the most economic substrate for the production of keratinase. After fermentation optimization, the recombinant *B. subtilis* did not improve much of its keratinase production, but *B. licheniformis* increased its production ten-fold. As a result, the production of keratinase by *B. licheniformis* is 4–5-fold less expensive than that by *B. subtilis*.

Discussion

Carbohydrate inhibition of keratinase expression was observed in both organisms (Table 1). This indicated that the host strain, *Bacillus subtilis* DB-104, has a catabolite repression regulatory mechanism common to other *Bacillus* spp [4,5,6,8,11,23,24]. The recombinant *B. subtilis* did not escape the carbohydrate inhibition that was found in *B. licheniformis*. However the *kerA* gene carried on a plasmid in *B. subtilis* was not affected by amino acids which down-regulated keratinase in *B. licheniformis* (unpublished data). Reducing the level of ground feathers in the medium improved keratinase production (Figure 1). It is possible that less free amino acids are released which represses *kerA*.

Studies demonstrated the importance of inoculum age on keratinase production by *B. licheniformis*. Improper starter culture age leading to poor product formation has been observed [22]. Lag phase of seed culture tends to prolong overall fermentation time due to insufficient activated cells. Bacterial inocula must be transferred in the log phase of growth, during which cells are still metabolically active [18]. Late inocula containing a high proportion of spores may be unable to revert to the vegetative cell cycle, and, consequently, limit the cell growth and product formation. The regulatory mechanism by which a late inoculum causes reduction of the keratinase yield by *B. licheniformis* is not known. Perhaps post-stationary phase inocula contain biological metabolites and amino acids that repress the keratinase gene. Possibly, sporulated bacteria could not effectively revert to the vegetative state when grown in minimal production medium containing insoluble feathers as the sole source of carbon and nitrogen. However, age of inoculum had little effect on *B. subtilis*.

Unlike *B. licheniformis*, the higher DO supply did not hamper the growth rate of *B. subtilis* (Figure 4), as is observed in aerobic procaryotes during fermentation [1,9,20,21,25,30–32,34]. However, keratinase production by both organisms declined at high DO levels (Figure 4). The mechanism of the negative effect of oxygen on keratinase is now known. The knowledge that neither organism needs excessive DO is beneficial for keratinase production, and therefore optimal fermentation can be maintained at moderate DO levels.

Lee *et al* [13] proposed that a temperature over 37°C

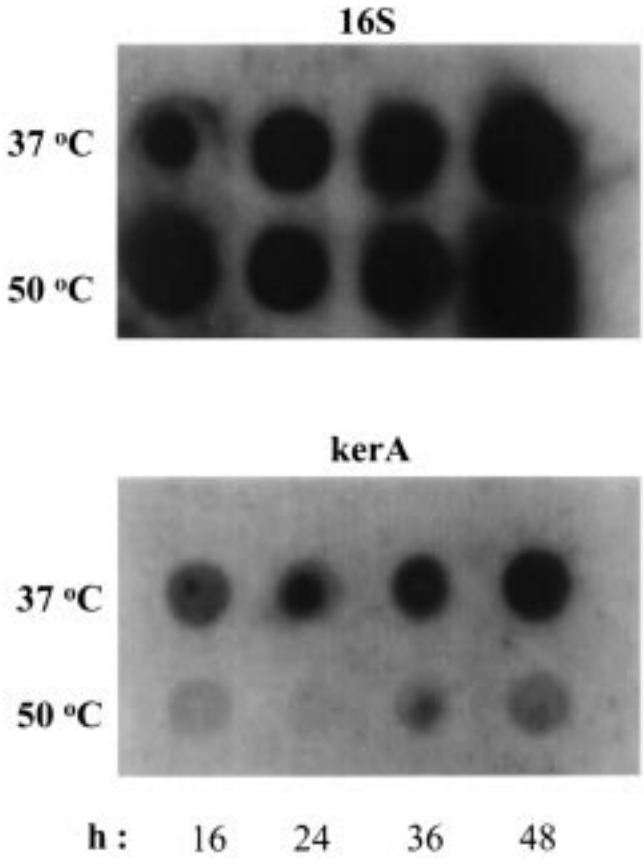

Figure 6 RNA dot blot analysis of *kerA* transcription level in *B. licheniformis* at different growth temperatures. Probed with DIG-16S as control and with DIG-*kerA*.

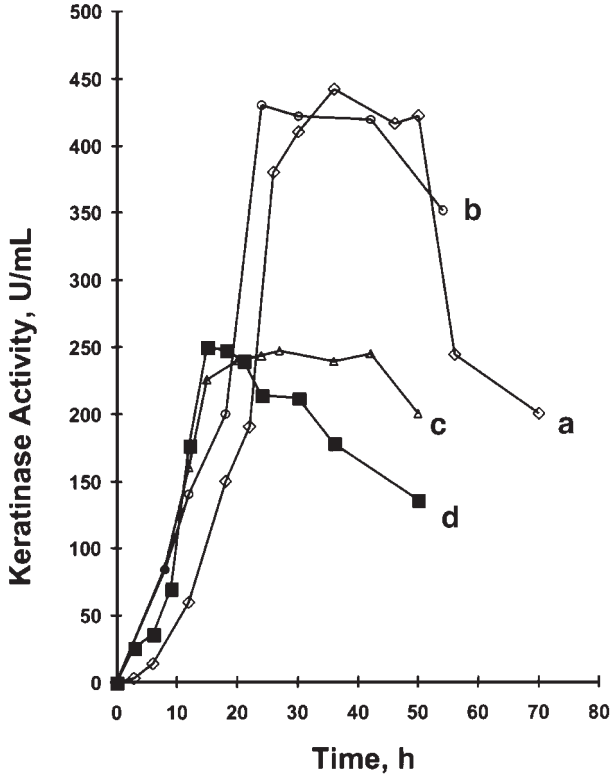

Figure 7 Production of keratinase by *B. licheniformis* at different modified procedures in 0.5% feather medium. (a) 37°C, no pH control. (b) 50°C, pH = 7.5 for 8 h, then cooled to 37°C without pH control. (c) 50°C, pH = 7.5 for 12 h, then cooled to 37°C without pH control. (d) 50°C, no pH control.

may promote replication of a thermostable plasmid vector as well as expression of cloned enzyme, while growth of host cells may be depressed at an elevated temperature. Production of amylase from a cloned thermostable plasmid was enhanced at an elevated temperature in a mesophilic *B. subtilis* host. In our cases, the maximum growth rate and keratinase productivity of *B. subtilis* occurred at 42°C instead of 37°C. However, the peak of keratinase activity was observed at 37°C. Elevated temperature increased cell growth but not enzyme production. The temperature differential effect on growth *vs* keratinase production was more obvious in *B. licheniformis*, where cells grow best at 50°C but keratinase production is best at 37°C. The high temperature may increase the protein turnover rate. The half-life of keratinase was 30 h at 37°C and 16 h at 50°C (data not shown). However, enzyme stability could only be considered a minor factor, because a much higher level of enzyme was produced at 37°C than at 50°C.

Kubo *et al* [10] reported that expression of genes from thermophilic bacteria are not always enhanced at elevated temperatures. Poor stability of the messenger RNA of keratinase at higher temperature was observed. Similar situations have been found in other *Bacilli* systems [23]. Alternatively, the *kerA* expression system may be heat-sensitive. From equal amounts of total RNA, mRNA of *kerA* was much lower in *B. licheniformis* cells at 50°C than at 37°C (Figure 6). Transcription of *kerA* is evidently heat-sensitive.

Production of keratinase by *B. subtilis* may be influenced by plasmid stability and copy number. Optimal cell growth would result in a higher number of gene copies due to multi-copies of the plasmid. That is why maximum cell density and enzyme activity are usually parallel at the same temperature. On the other hand, the replication of initiation frequency of plasmid DNA increases with a decrease in specific growth rate of recombinant cells and repressor proteins [14]. Other studies found similar results [3,7,27,29]. This supports the evidence related to maximum bulk enzyme activity observed at 37°C instead of 42°C, at which maximum growth rate occurred.

In flask cultures, at 37°C *B. subtilis* over-expressed keratinase by 3–4-fold compared with *B. licheniformis* at 50°C [17]. After optimizing fermentation conditions as described in this report, however, an opposite result was observed. As shown in Figure 5 and Table 3, *B. licheniformis* maintained a higher enzyme yield and a much better productivity.

In summary, a novel scale-up procedure for the fermentation production of keratinase from *B. licheniformis* PWD-1 was developed. However, this study demonstrated that both the parent and recombinant strains need rigorous optimization and characterization processes during scale-up fermentation production of the enzyme.

Table 3 Comparison of fermentation performance at different scales[a]

Culture vessel	*B. licheniformis*						*B. subtilis*		
	Temperature shift[b]			37°C			37°C		
	Activity (U ml^{-1})	Time (h)	Productivity (U ml^{-1} h^{-1})	Activity (U ml^{-1})	Time (h)	Productivity (U ml^{-1} h^{-1})	Activity (U ml^{-1})	Time (h)	Productivity (U ml^{-1} h^{-1})
50-ml Flask	–	–	–	430 ± 33	40	10.75	306 ± 11	62	4.94
15-L Fermentor	440 ± 36	24	18.33	450 ± 28	30	15.00	310 ± 16	48	6.46
150-L Fermentor	449 ± 44	24	18.71	489 ± 30	30	16.30	304 ± 17	48	6.33

[a]For *B. licheniformis*: 0.5% feather medium, 10% DO in fermentors and no pH control. For *B. subtilis*: 1.0% feather medium, 20% DO in fermentors and pH control at 8.0.
[b]Temperature shift: 8 h at 50°C followed by 16 h at 37°C.

Table 4 Estimation of fermentation production cost of keratinase with three different substrates

Substrate	Keratinase activity (U ml^{-1})	Cell yield (g g^{-1} substrate)	Keratinase yield based on biomass[b] (mg g^{-1} cells)	% Keratinase in total soluble protein	Crude keratinase yield (g L^{-1})	Substrate cost factor[c]	Relative cost[d] (%)
			B. licheniformis				
Ground feathers	450 ± 15	0.31	48.3	13.0	0.7	1.0	100
Commercial feather meal	510 ± 22	0.32	54.8	14.7	0.7	1.0	106
Soy flour	475 ± 18	0.32	50.9	14.2	0.6	1.5	142
			B. subtilis				
Ground feathers	305 ± 8	0.33	15.2	12.0	0.4	1.0	472
Commercial feather meal	301 ± 11	0.34	15.0	11.9	–	1.0	519
Soy flour	325 ± 18	0.35	16.2	12.8	–	1.5	581

[a]Results for *B. licheniformis* obtained from the average of 10-L and 150-L fermentors controlled at 37°C with optimal cultivation conditions. Experiments involving *B. subtilis* were employed in a 10-L fermentor controlled at pH = 8.0, 37°C, and other optimal conditions.
[b]Specific activity: 5990 U mg^{-1} [15].
[c]Cost factor estimated by current market price for commercial feather meal and soy flour compared with feathers. Ground feathers = $0.32/kg (assumed to be equal to commercial feather meal). Commercial feather meal = $0.32/kg. Soy flour = $0.47/kg.
[d]Relative cost calculated based on substrate keratinase productivity (U g^{-1} substrate h^{-1}).

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