SHORT COMMUNICATION

Isolation and screening of D-amino acid amidase producing bacteria from soil samples

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Abstract

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Isolation and screening of D-amino acid amidase producing bacteria from fifty-four soil samples taken on Hat Yai campus of Prince of Songkla University were conducted using acclimation culture technique in medium broth containing D-phenylalanine amide as a sole source of nitrogen. Bacteria capable of growing at 30ºC and 45ºC were screened and isolated. Twenty-one and seventeen soil samples showed the hydrolysis of D-phenylalanine amide to D-phenylalanine determined by thin layer chromatography and forty-seven and thirty-four strains were isolated at 30ºC and 45ºC, respectively. Each isolate obtained was screened for its ability to degrade D-phenylalanine amide. Thirteen of forty-seven strains and nineteen of thirty-four strains isolated at 30ºC and 45ºC, respectively exhibited D-phenylalanine amide degradation. However, only isolate BS16 exhibited degradation toward D-tert-leucine.

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The enzyme activity toward D-phenylalanine amide was detected from the bacterial cells, but not in supernatant indicating that the amidase was an intracellular enzyme. The bacterial cells grown in the broth with and without D-amino acid amide showed enzyme activity leading to the conclusion that it was constitutive enzyme.

Key words : bacteria from soil, D-amino acid amidase producing bacteria, D-phenylalanine amide

บทคัดย่อ

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การแยกและการคัดเลือกแบคทีเรียที่ผลิตเอนไซม์ดีอะมีโนเอซิดอะมิเดสจากตัวอย่างดิน

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การแยกและคัดเลือกจุลินทรีย์ที่สามารถผลิตเอนไซม์ดีอะมิโนเอซิดอะมิเดสจากตัวอย่างดิน จำนวน 54 ตัวอย่างที่เก็บจากพื้นดินในบริเวณมหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา โดยใช้เทคนิคการทำให้เชื้อคุ้นเคย (acclimation culture technique) ในอาหารเลี้ยงเชื้อที่มี D-phenylalanine amide เป็นแหล่งไนโตรเจนเพียงชนิดเดียว ที่อุณหภูมิ 30 และ 45°C พบตัวอย่างดินจำนวน 21 และ 17 ตัวอย่าง ที่สามารถย่อย D-phenylalanine amide ไปเป็น D-phenylalanine ได้ที่อุณหภูมิดังกล่าว ตามลำดับ โดยตรวจสอบความสามารถในการย่อยด้วยวิธี thin layer chromatography เมื่อทำการแยกแบคทีเรียจากตัวอย่างดินเหล่านี้สามารถแยกแบคทีเรียได้ 47 และ 34 สายพันธุ์ ที่อุณหภูมิ 30 และ 45°C ตามลำดับ จุลินทรีย์ที่แยกได้เหล่านี้ถูกนำมาคัดเลือกโดยตรวจสอบความสามารถในการย่อย D-phenylalanine amide ซึ่งพบว่ามีอยู่ 13 สายพันธุ์ จาก 47 สายพันธุ์ และ 19 สายพันธุ์ จาก 34 สายพันธุ์ที่แยกที่อุณหภูมิ 30 และ 45°C ตามลำดับ แสดงคุณสมบัติในการย่อย D-phenylalanine amide อย่างไรก็ตามมีเพียงแบคทีเรียที่แยกจากตัวอย่างดิน (BS16) ที่แยกได้ที่อุณหภูมิ 30°C เท่านั้นที่สามารถย่อย D-tert-leucine amide ได้

กิจกรรมของเอนไซม์ตรวจพบเมื่อใช้เซลล์แบคทีเรียในการทำปฏิกิริยาแต่ไม่พบกิจกรรมของเอนไซม์เมื่อใช้ส่วนใสของอาหารเลี้ยงเชื้อที่แยกเซลล์แบคทีเรียออกแล้ว ซึ่งแสดงว่าแบคทีเรียไม่หลั่งเอนไซม์ชนิดนี้ออกนอกเซลล์ เมื่อเลี้ยงแบคทีเรียในอาหารที่มี และไม่มี D-amino acid amide พบว่าแบคทีเรียสามารถผลิตเอนไซม์ได้ทั้งในสภาวะที่มีและไม่มี D-amino acid amide ซึ่งนำไปสู่ข้อสรุปได้ว่าการผลิตเอนไซม์อะมิเดสของแบคทีเรีย สายพันธุ์ BS16 ไม่จำเป็นต้องมีสาร D-amino acid amide ไปเหนี่ยวนำ

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D-amino acids are important chiral building blocks for the production of pharmaceuticals, food additives, herbicides and other agrochemicals. They are used as intermediates for the preparation of β-lactam antibiotics such as semi-synthetic cephalosporins and penicillins (Yamada and Shimizu, 1988). D-alanine can be used in the synthesis of alitame (synthetic sweetener) (Glowaky *et al*., 1991). Substitution of D-amino acids to their L-counterparts in the synthesis of biologically active compounds such as peptide hormones or antibiotics could lead to metabolically stable and long-acting properties (Asano and Lubbehusen, 2000). However, there are few industrial processes for producing these bioactive enantiomers. Therefore, enzymes involved in the nitrile metabolism from

microbial source are receiving great attention. Nitriles are converted to their corresponding acids by two biological pathways (Ciskanik *et al*., 1995). In one pathway, nitrilases are required to yield the corresponding carboxylic acids and ammonia:

$$RCN + 2H_2O \rightarrow RCOOH + NH_3$$

The other pathway is a two-step pathway, in which nitriles are converted into the corresponding amides by nitrile hydratase and amides are hydrolyzed to the corresponding carboxylic acids by amidases:

$$RCN + H_2O \rightarrow RCONH_2$$
$$RCONH_2 + H_2O \rightarrow RCOOH + NH_3$$

Most L-amino acids are at present produced by fermentation, whereas D-amino acids are synthesized chemically and enzymatically. However, enzyme-catalyzed methods have offered greater benefit than the chemical synthesis, especially the discovery of D-stereospecific enzymes (Asano and Lubbehusen, 2000).

D-amino acid amidases are increasingly being recognized as an important catalysts in stereospecific production of D-amino acid. They catalyze the stereospecific hydrolysis of D-amino acid amide to yield D-amino acid and ammonia. A few D-amino acid amidases have been reported so far. D-amino acid amidase from *Arthrobacter* sp. NJ-26 was discovered to be specific to D-alanine amide (Ozaki *et al*., 1992). (R)-Enantioselective amidase secreted by *Commamonas acidovorans* was purified, characterized and cloned by Hayashi *et al*. (1997). However, its stereospecificity was quite low. The strict D-stereospecific amino acid amidase from *Ochrobactrum antropi* SV3 was investigated by Asano *et al*. (1989), and later was cloned, purified and characterized (Komeda and Asano, 2000). The enzyme was remarkably active on D-phenylalanine amide, D-tyrosine amide and D-tryptophan amide.

Here, we report on the success of isolation and screening of D-amino acid amidase producing bacteria from soil samples collected on Hat Yai campus of Prince of Songkla University.

Materials and Methods

Chemicals

D-Phenylalanine amide HCl, D-tert-leucine amide HCl, D-tert-leucine and D-phenylalanine were kindly provided by Professor Yasuhisa Asano (Toyama Prefectural University, Japan). They were synthesized as described by Asano *et al*. (1989 and 1996). Cromatofolios AL TLC 20x20 cm Silica gel 60 F254 was purchased from Merck (Germany). Nicotinic acid, thiamine-HCl, pyridoxine HCl, riboflavin and folic acid were supplied by Wako Pure Chemical Industries, Ltd. (Japan).

Isolation and Screening of D-amino acid amidase producing bacteria

Microorganisms, possessing an ability to utilize D-phenylalanine amide as nitrogen source, were isolated from soil by an acclimation culture technique (Asano *et al*., 1989). One gram of soil sample was added into 2 ml of enrichment broth containing 5 g of glycerol, 2 g of D-phenylalanine amide HCl, 2 g of K_2HPO_4, 1 g of NaCl, 0.2 g of $MgSO_4.7H_2O$, 0.5 g of yeast extract, 0.02 µg of biotin, 4 µg of calcium pantothenate, 20 µg of inositol, 4 µg of nicotinic acid, 4 µg of thiamine HCl, 2 µg of pyridoxine HCl, 2 µg of p-aminobenzoic acid, 2 µg of riboflavin, and 0.1 µg of folic acid in 1 liter of tap water, pH 7.0. D-phenylalanine amide HCl was filter-sterilized and added to the autoclaved-mixture (Asano *et al*., 1989). Soil samples were aerobically incubated with the medium at 30ºC and 45ºC, 250 rpm for 24 h. Ten µl of the culture was then transferred into 2 ml of fresh medium, and was further incubated at 30ºC and 45ºC for a week. The formation of D-phenylalanine in the culture broth was detected daily by thin layer chromatography. Bacteria were then isolated from the positive tubes by subculturing on the enrichment agar (1.6 % agar added into enrichment broth mentioned above) to obtain single colonies. The colonies were picked according to the morphological characteristics on the agar plate. Each pure isolate was then cultured in the

enrichment broth at its isolating temperature for one week, and tested daily for its ability to degrade D-phenylalanine amide by spotting 1 *µl* of culture broth on TLC. The bacterial isolates exhibiting D-phenylalanine amide degradation were kept in agar slants of the enrichment medium described above at temperature of 4ºC, and were transferred monthly. All selected isolates were also tested for the ability to degrade D-tert-leucine amide. The isolates which caused degrade both D-phenylalanine amide and D-tert-leucine amide were selected for further study.

Determination of D-amino acid amidase activity

The presence of D-amino acid amidase activity was detected by the occurrence of a band of either D-phenylalanine or D-tert-leucine, which appeared at the lower R_f compared to its amide counterpart on TLC due to its higher polarity. To prove whether the enzyme production was inducible or constitutive, the D-amino acid activity of the bacterial cells exhibiting both D-phenylalanine amide and D-tert-leucine amide was determined using supernatant and cells cultured in TGY broth (5 g of tryptone, 5 g of yeast extract, 1g of K_2HPO_4 and 1 g of glucose in 1 liter of tap water) and the medium broth containing either D-phenylalanine amide or D-tert-leucine amide as described above. The cells were grown in three types of medium broth by shaking at 250 rpm at 30ºC or 45ºC for 24 h, and were then centrifuged at 4000xg for 30 min. The bacterial cells were washed twice with 0.9% saline solution. The supernatant and the washed cells were then determined for the presence of D-amino acid amidase activity.

Enzyme activities of bacterial cells and cell-removal culture broth were determined by performing enzyme reaction. The reaction mixture contained 500 µl of 100 mM D-phenylalanine amide in 0.1 M Tris-HCl (pH 8.0), 400 µl of 0.1 M Tris-HCl (pH 8.0) and 100 µl of bacterial cell suspension or cell-removal culture broth (supernatant). The reaction was performed at 30ºC for 6 h. The reaction mixture was drawn hourly, and determined for degradation of D-phenylalanine amide by TLC.

Determination of D-phenylalanine amide degradation by TLC

The degradation of D-phenylalanine amide was detected by the formation of D-phenylalanine, which migrated on TLC at a lower R_f than its amide counterpart. The TLC plate was developed in the solvent mixture containing *n*-propanol: ammonia (8:1 by vol). The degradation of D-tert-leucine was detected in the same manner by developing TLC plate in the solvent mixture of water: *n*-propanol:ammonia (1:8:1 by vol). The plate was then dried and visualized by spraying 0.2% ninhydrin in 0.1 M citrate buffer (pH 5.0), followed by mild heating.

Results and Discussion

Isolation for D-amino acid amidase-producing bacteria

Fifty-four soil samples were taken from various places around PSU campus depending on the appearance and location. They were aerobically incubated at 30ºC and 45ºC in the medium broth containing D-phenylalanine as a sole nitrogen source. After the second transfer to the same medium broth, the ability to degrade D-phenyalalnine amide was determined by TLC. The spot of D-phenylalanine was appeared at lower Rf (0.64-0.69) as indicating by an arrow as shown in Figure 1, when the degradation occurred. This was due to its lower polarity, compared to its amide derivative. Twenty out of fifty-four soil samples (37%) were negative, and twenty-four (63%) were positive. Twenty-one and seventeen soil samples showed the degradation ability at the incubation temperatures of 30 and 45ºC, respectively (Table 1), indicating 39% and 32% by chance to obtain the positive soil samples at 30 and 40ºC using acclimation technique.

Only four soil samples (7%) showed positive result at both 30 and 45ºC, and the rest (93%) showed the degradation ability at either at 30 or 45ºC, indicating that most of the degradation that occurred was caused by different groups of bacteria grown at various temperatures. There was low

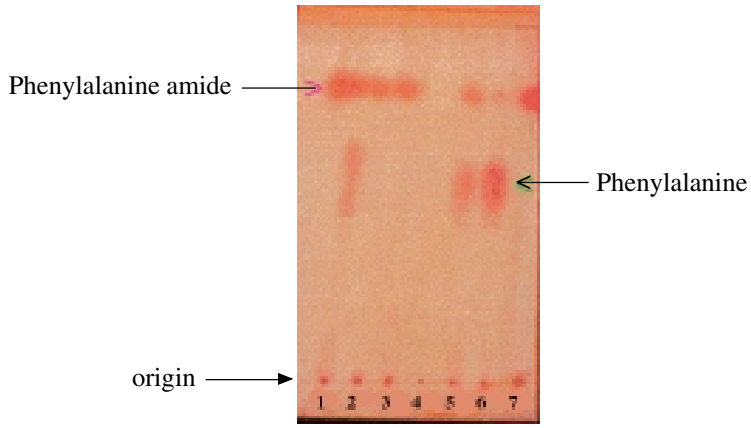

Figure 1. Thin layer chromatography profile of D-phenylalanine amide broth cultured with soil samples (see Materials and Methods for details); S11 (lane 2), S12 (lane 3), S13 (lane 4), S14 (lane 5), S15 (lane 6), S16 (lane 7) incubated at 45ºC. Lane 1 is standard D-phenylalanine amide.

possibility to obtain the positive result for rich soil samples, which was mixed with decomposed plants and animals (S7, S40, S48). As shown in Table 1, 63% of soil samples exhibited the ability to react with D-amino acid amide. This is not surprising as D-form amino acids can be found as the important component of bacterial cells, particularly bacterial peptidoglycans (Madigan *et al*., 1997).

Bacterial strains were isolated from positive soil samples by their physical appearance on agar plate containing D-phenylalanine amide as a sole source of nitrogen. Forty-seven and thirty-four strains were isolated at temperature of 30 and 45ºC, respectively (Table 1). Morphologically, all bacteria isolated at 30ºC were totally different from those isolated at 45ºC although they are from the same soil samples, particularly soil sample S10, S14, S30 and S50. This finding supported the hypothesis mentioned above. In fact, there was no bacterial strain obtained at 45ºC from soil sample

Table 1. The ability of D-phenylalanine amide degradation of soil samples incubated at 30ºC and 45ºC; (a), number of isolates obtained, (b) number of of isolates possessed the degradation ability.

Soil sample	Appearances	Degradation of D-phenylalanine amide		Numbers of Isolates obtained	
		30ºC	45ºC	30ºC	45ºC
S1	moist, red	+	-	1[a](0[b])	-
S2	moist, crumble	+	-	3(1)	-
S3	moist, coarse, sandy	-	+	-	2(2)
S4	coarse, red	+	-	1(0)	-
S5	crumble, black	-	-	-	-
S6	moist, fine, black	-	+	-	2(1)
S7	moist decomposed	-	-	-	-
S8	decomposed, black	-	+	-	1(1)
S9	crumble, red	-	+	-	1(1)
S10	brown, sticky	+	+	1(0)	1(1)

to be continued

Table 1. (continued)

Soil sample	Appearances	Degradation of D-phenylalanine amide		Numbers of Isolates obtained	
		30ºC	45ºC	30ºC	45ºC
S11	fine, light brown	-	+	-	5(5)
S12	coarse, dark brown	-	-	-	-
S13	coarse, light brown	-	-	-	-
S14	fine, brown, sticky	+	+	1(0)	-
S15	crumble, red-brown	-	+	-	2(1)
S16	red, sticky	+	-	1(1)	-
S17	hard, dark brown	+	-	2(1)	-
S18	crumble brown	+	-	1(0)	-
S19	muddy	+	-	2(1)	-
S20	moist, airy	+	-	2(0)	-
S21	fine, crumble, light-brown	+	-	2(0)	-
S22	moist, brown, sticky	+	-	4(1)	-
S23	moist, brown, sandy	+	-	2(1)	-
S24	termite brown	+	-	1(0)	-
S25	moist, fine, sticky	-	-	-	-
S26	moist, fine	+	-	2(0)	-
S27	hard, dark-brown	-	+	-	1(1)
S28	clump, dark brown	-	-	-	-
S29	moist, fine, light-brown	-	-	-	-
S30	crumble, coarse, brown	+	+	2(2)	2(0)
S31	moist, fine, dark-brown	+	-	1(1)	-
S32	moist, coarse, sandy	-	+	-	2(2)
S33	coarse, sandy, white-brown	-	-	-	-
S34	moist, fine, brown	-	-	-	-
S35	coarse, red	-	+	-	2(2)
S36	fine, dark brown	-	+	-	4(2)
S37	moist, fine brown	+	-	4(1)	-
S38	moist, sticky, red	-	-	-	-
S39	fine, light brown	-	-	-	-
S40	decomposed root, black	-	-	-	-
S41	coarse, black	-	-	-	-
S42	moist, fine, black	+	-	4(2)	-
S43	coarse, red-brown	-	-	-	-
S44	coarse, red	-	-	-	-
S45	coarse, light brown	+	-	4(1)	-
S46	coarse, red	-	-	-	-
S47	coarse, black (red mixed)	-	+	-	2(0)
S48	decomposed, fine, black	-	+	-	2(0)
S49	moist, fine, light-brown	-	+	-	2(0)
S50	coarse, dry, brown	+	+	2(0)	3(0)
S51	fine, crumble, black	-	-	-	-
S52	moist, coarse, gray	+	-	4(0)	-
S53	moist, sandy, light-brown	-	-	-	-
S54	moist, coarse, black	-	-	-	-

S14, although the soil sample showed a positive result. This could be due to the limitation of the bacterial growth on the isolating media. Using enrichment media for the isolation may provide greater opportunity to obtain more fastidious microbes, yet it could also enhance the growth of highly competitive one, which could dominate in the culture, particularly those that can grow in the presence of trace amounts of nitrogen provided by yeast extract.

Screening for D-amino acid amidase-producing bacteria.

Each individual isolate was tested for the ability to degrade D-phenylalanine amide as the primary screening criteria. Thirteen strains degrading D-phenylalanine amide to D-phenylalanie were screened at 30ºC, whereas nineteen isolates were selected at 45ºC. The low number of positive isolates obtained supported the above discussion. This indicated that this group of bacteria was not high competitors in the term of nutrients.

According to morphological study on the positive isolates, most isolates (92%) obtained at 30ºC were gram-negative, 46% were rod shape, and 54% were cocci (Table 2). At 45ºC, 84% of the selected isolates appeared to be rod shape, and 58% were gram-negative (Table 3). Gram-positive, rod shape bacteria were isolated in greater numbers than gram-negative ones, corresponding to the fact that most of gram-positive, rods are spore-formers, which are more tolerable to harsh environments in the soil, such as dryness and heat (Madigan *et al*., 1997).

Secondary screening was performed on the ability to D-tert-leucine amide dagradation. All D-amino acid amidases reported so far are unable to react specifically on D-tert-leucine amide. Plus, D-tert-leucine is hard to obtain, even with fine chemical procedure. Most D-amino acids and their derivatives are not available commercially. This is why there are only a few groups of scientists working on these D-enantioselective enzymes. Enantioselective amidase from *Comamonas acidovorans* KPO-2771-4 was specific to phenylalanine amide, leucine amide, but its specificity toward D-configuration was not high (Hayashi *et al*., 1997). D-Amino acid amidase from *Ochrobactrum anthropi* SV3 showed highly stereospecific activity toward D-configuration, preferentially aromatic amino-acid amide (Komeda and Asano, 2000). In addition, D-alaninamide amidase from *Arthrobacter* sp. was purified and used for the production of D-alanine (Ozaki, *et al*., 1992). Ciskanik *et al*. (1995) reported

Table 2. Morphological characteristics of D-phenylalanine-amide-degrading bacteria isolated at 30ºC

Bacterial Isolates	Morphological characteristics	
	Cultural	Microscopic
BS2	Corrugated, rough, white	Gram-negative, rod
BS16	Round (1mm), smooth, glossy, beige color	Gram-negative, rod
BS17	Round (2 mm), opaque yellow	Gram-negative, rod
BS19	Round (3 mm), opaque white	Gram-negative coccus
BS22	Irregular (5-6 mm), white	Gram-negative, coccus
BS23	Round (1 mm), smooth, yellow	Gram-negative, rod
BS30A	Round (3 mm), yellow, mucilaginous	Gram-negative, coccus
BS30B	Round (1 mm), smooth, white	Gram-negative, rod
BS31	Round (1 mm), opaque white	Gram-negative, coccus
BS37	Round (1 mm), bright yellow	Gram-negative, coccus
BS42A	Round (1 mm), white	Gram-negative, coccus
BS42B	Round (2 mm), orange-yellow	Gram-positive, rod
BS45	Round (2 mm), mucilaginous, opaque white	Gram-negative, coccus

Table 3. Morphological characteristics of D-phenylalanine-amide-degrading bacteria isolated at 45ºC

Bacterial Isolates	Morphological characteristics	
	Cultural	Gram staining
BS3A	Corrugated, white	Gram-positive, rod
BS3B	Corrugated, glossy	Gram-positive, rod
BS6	Round (2 mm), opaque white	Gram-negative, rod
BS8	Corrugated, glossy	Gram-negative, rod
BS9A	round (1 mm), opaque white,	Gram-negative rod
BS9B	mucilaginous	
BS10	Corrugated, white, nipple	Gram-positive, rod
BS11A	Round (3 mm), smooth, glossy	Gram-positive, rod
BS12B	Round (3 mm), smooth, opaque white	Gram-positive, rod
BS13C	Corrugated, opaque white	Gram-positive, rod
BS14D	Corrugated, opaque white	Gram-negative, rod
BS15F	Round (3 mm), smooth, glossy	Gram-positive, rod
BS15	Irregular (5-6 mm), opaque white, nipple	Gram-negative, rod
BS27	Round (1 mm), opaque white	Gram-negative, rod
BS32A	Round (1 mm), red	Gram-positive, coccus
BS32B	Irregular (5-6 mm), opaque white	Gram-negative, coccus
BS35A	Irregular (5-6 mm), glossy	Gram-negative, rod
BS35B	Irregular (5-6 mm), opaque white	Gram-negative, rod
BS36A	Round (4 mm), beige, nipple	Gram-negative, rod
BS36B	Round (3 mm), opaque white	Gram-negative, coccus

an enantioselective amidase from *Pseudomonas chlororaphis* B23, which preferred organic acid amide to DL-phenylalanine amide. However, there is no report whatsoever regarding D-amidase specific to D-tert-leucine.

The secondary screening was conducted by growing all positive bacterial isolates in medium containing D-tert-leucine amide as a sole source of nitrogen. The degradation of D-tert-leucine amide to D-tert-lucine was detected by TLC. Unfortunately, all nineteen strains isolated at 45ºC were not capable of degrading D-tert-leucine amide. Only strain BS16 growing at 30ºC was found to exhibit the ability to degrade both D-phenylalanine amide and D-tert-leucine amide as shown in Figure 2. The concentration of D-tert-leucine amide was obviously reduced as fading bands (upper) of substrate were revealed, while the product band (lower) became intense. Morphological study showed that this strain appeared as a round (1 mm), smooth, entire, glossy colony with beige color on agar plate. It was shown to be motile, non-spore-forming and gram-negative rod shape.

Enzyme activity of D-amino acid amidase from strain BS16

Isolate BS16 was grown in both medium broths, one containing D-phenylalanine amide and the other containing D-tert-leucine amide, at 30ºC for 24 h. The bacterial cells were removed from the broth (supernatant), washed and suspended in 0.1 M Tris-HCl (pH 8.0). The enzyme activities of cell suspension and supernatant were evaluated by performing the reaction as described above. The cell reaction was performed based on whole-cell reaction using either D-phenylalanine amide or D-tert-leucine amide as substrate. D-Phenylalanine was detected in the reaction mixture with cell suspension after 1 h of reaction and the intensity

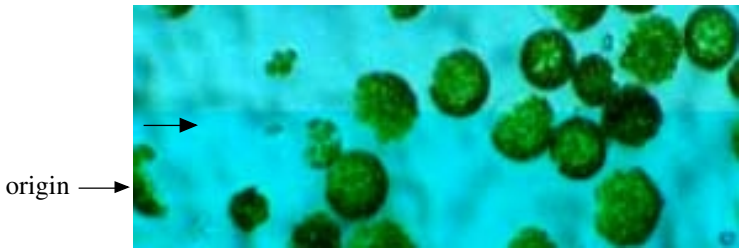

Figure 2. TLC profile of D-tert-leucine amide-containing broth cultivated with 13 bacterial strains isolated at 30ºC; strain BS2 (lanes 2,3), strain BS16 (lanes 4, 5), strain BS17 (lanes 5, 6), strain BS19 (lanes 7, 8), strain BS22 (lanes 7, 8), strain BS23 (lanes 11, 12), strain BS30A (lanes 13, 14), strain BS30B (lanes 15, 16), strain BS30C (lanes 17), strain BS31 (lane s20, 21), strain BS37 (lanes 22, 23), strain BS42 (lanes 24, 25), strain BS45 (lane 26), standard D-tert-leucine amide (lanes 1, 10, 19), standard D-tert-leucine (lanes 9, 18, 27). Only strain 16 showed degradation of D-tert-leucine as the product bands appeared (arrow).

of the product band increased after 2 and 4 h. On the contrary, the supernatant obtained from both cultures did not show any degradation, indicating that D-amino acid amidase was secreted intracellularly by isolate BS16. The result correlated with previous studies that most microbial amidase enzymes were retained in the cells (Asano *et al*., 1989; Ciskanik *et al*., 1995; Mayaux *et al*., 1990; Ozaki *et al*., 1992). The enzyme from *Ochrobactrum anthropi* recognized amide bond of D-amino acid as well as D-peptides, and it was produced intracellularly (Asano *et al*., 1989), while the one produced by *Bacillus subtilis* was an extracellular enzyme (Cheggour *et al*., 2000). This study on whole-cell reaction could provide information useful for further steps of experiment in enzyme purification, substrate specificity and characterization of the purified enzyme. Interestingly, cells grown in D-phenylalanine amide showed much lower activity, compared to cells grown in D-tert-leucine amide containing broth. This observation may be simply explained that this enzyme showed low activity toward D-tert-leucine as mentioned above. In order to survive in the broth containing D-tert-leucine as sole nitrogen source, bacteria need to produce higher amount of enzyme hence its higher activity in the whole cell reaction.

It required more than 4 h to detect slight D-tert-leucine band, indicating low activity of the enzyme toward D-tert-leucine amide. The fading bands appearing from the reaction of the supernatants were caused by the D-amino acids remaining in the supernatant. The amino acid bands appearing at 0 h confirmed this conclusion.

Bacterial cells grown in TGY (without amino acid amide) showed degradation activity toward D-phenylalanine (Figure 4). It could be concluded that the enzyme was produced constitutively because the enzyme activity was detected in the cells grown in TGY as well as both D-amino acid amide containing broth. The bacteria did not require D-amino acid amide to induce the production of the enzyme, which was similar to D-amidase produced by *Ochrobactrum anthropi* (Asano *et al*., 1989). However, amidase enzymes from *Rhodococcus* (Mayaux *et al*., 1991), *Pseudomonas chlororaphis* B23 (Ciskanik *et al*., 1995) were reported as inducible enzymes.

Data shown in Figure 4 support the conclusion above that the enzyme was retained in the cell as bacterial cells growing in TGY media showed the formation of D-phenylalanine, whereas supernatants from the culture broth did not show any enzyme activity. The results indicated that strain BS16 produced D-amino acid amidase enzyme constitutively and intracellularly as there was no enzyme activity in the supernatant. This finding is very important for further study for

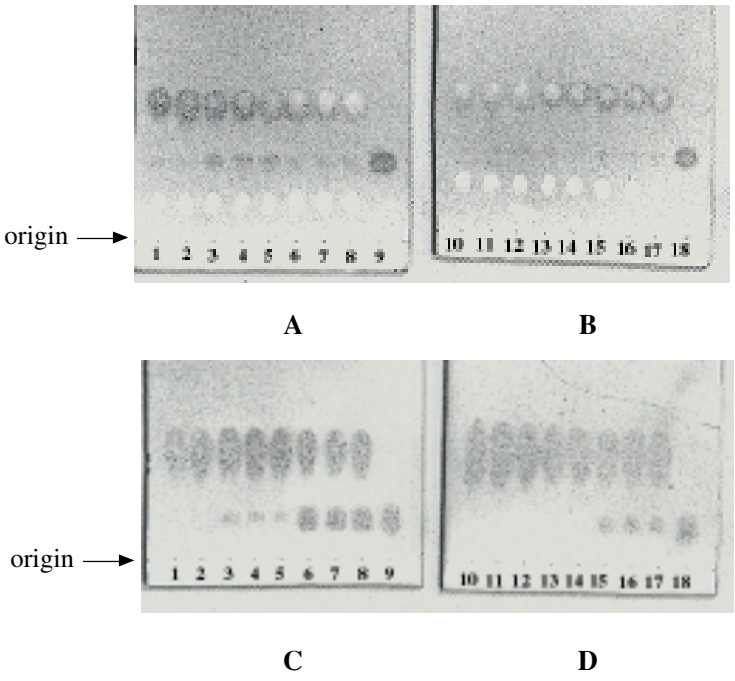

Figure 3. TLC profiles of reaction mixtures containing, D-tert-leucine amide (panel A, B); and D-phenylalanine amide (panels C, D) as substrates. On panels A and C, the reactions were performed by adding either bacterial cells (lanes 6, 7, 8) or supernatant (lanes 3, 4, 5) from BS16 grown in D-tert-leucine amide containing broth. On panels B and D, the reactions were performed by adding either bacterial cells (lanes 15, 16, 17) or supernatant (lanes 12, 13, 14) from BS16 cultured in D-phenylalanine amide containing broth. Lanes1, 2, 10, 11 of panels A, B are standard D-tert-leucine amide; and those of panel C, D. Lanes 9, 18 of panels A, B are standard D-tert-leucine, whereas those of panels C, D are standard D-phenylalanine.

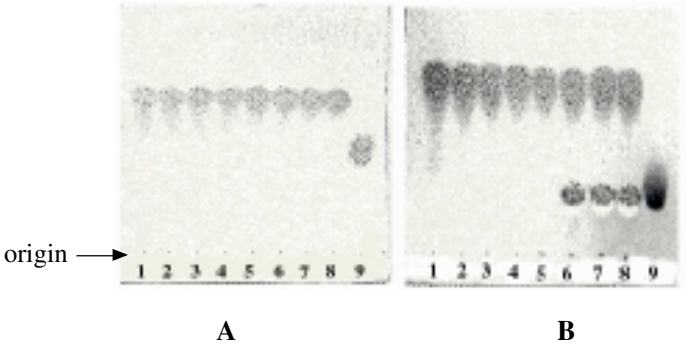

Figure 4. TLC profiles of reaction mixtures containing; D-tert-leucine amide (panel A), and D-phenylalanine amide (panel B) as substrates. The reactions were performed by adding either bacterial cells (lanes 6, 7, 8) or supernatant (lanes 3, 4, 5) from BS16 cultivated in TGY broth. Lanes 1, 2 panel A are standard D-tert-leucine amide, and those of panel B are standard D-phenylalanine amide. Lane 9 of panel A is standard D-tert-leucine, and that of panel B is standard D-phenylalanine.

enzyme preparation and purification because the bacterial cells can be cultivated in TGY instead of D-amino acid amide containing media since D-form of amino acids cannot be obtained commercially. Bacterial cells can be harvested, washed and resuspended in buffer solution before cell disruption to obtain cell-free extract for the enzyme purification. So far, the purification, characterization and substrate specificity (D-stereospecificity) of D-amino acid amidase from strain BS16 are still under investigation.

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