RESEARCH ARTICLE

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Coastal Soil Actinomycetes: Thermotolerant Strains Producing *N*-Acylamino Acid Racemase

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ABSTRACT: Sixty four soil samples collected from the coastal ecosystem of Chonburi, Rayong and Trat Provinces were isolated for thermotolerant actinomycetes. Among 567 isolates of thermotolerant strains, 12 appeared to be *N*-acylamino acid racemase producers at various quantities. From the 16S DNA gene sequence and morphological characteristics strain Sal35-16, the highest enzyme producer, was identified as *Streptomyces tendae*. By measuring the end product, this *S. tendae* could produce L-methionine at 0.68 mM. Another high enzyme producer, strain Sal31-15, identified as *Streptomyces maritimus*, produced 0.21 mM L-methionine and 5.79 mM D-methionine. Both strains had spiral spore in chains with warty spores. Strain Sal35-16 and strain Sal31-15 grew well between 30-50 °C and 30-45 °C, respectively. Both strains were capable of producing *N*-acylamino acid racemase, not previously known from these species of *Streptomyces.*

KEYWORDS: Actinomycetes, *Streptomyces, N*-acylamino acid racemase.

INTRODUCTION

Amino acid racemase enzyme now takes an important role in the industrial sector.[1] In an industrial process, L-methionine is continuously being produced from *N*- acetyl-DL-methionine by the action of the immobilized of L-aminoacylase. However, only *N*-acetyl-L-methionine can be converted into L-methionine, leaving some amount of *N*-acetyl-D-methionine.[2] A thermotolerant or thermostable enzyme is needed for the complete conversion of *N*-acetyl-DL-methionine into L-methionine. *N*-acylamino acid racemase appears to be the enzyme. Tokuyama *et al*[1] discovered that, of approximately 49,000 strains of bacteria, actinomycetes, yeast and fungi, only some strains of actinomycetes could produce *N*-acylamino acid racemase. These microbes included *Actinomadura roseoviolacea* IFO 14098, *Actinomyces aureomonopodiales* IFO 13020, *Jensenia canicruria* IFO 13914, *Amycolatopsis orientalis* IFO 12806, *Sebekia benihana* IFO 14309, *Streptomyces coelescens* IFO 13378, *S. celluloflavus* IFO 13780, *S. alboflavus* IFO 13196, *S. aureocirculatus* IFO 13018, *S. diastatochromogenes* IFO 13389, *S. spectabilis* IFO 13424, *S. tuirus* IFO 13418, and *S. griseoaurantiacus* IFO 13381. Moreover, there are more strains of actinomycetes in natural soils for which it still unknown whether they are enzyme producers. [3]

MATERIALS AND METHODS

Isolation:

Soil samples were collected in various coastal areas in Chonburi, Rayong and Trat Provinces. Before isolation, the sample soils were pretreated at 55 °C for 15 min and at 100° C for 60 min. One gram of each soil was suspended in 9 ml peptone water solution, diluted and spread on Actinomycetes Isolation Agar and Starch Casein Agar plates, then incubated at 40 °C for 7-10 days.

Morphological Characteristics:

Spore chains and hyphae were determined under light microscope with both the culture plates and coverslips inserted into the agar according to Williams *et al*[4]. Scanning electron microscopy was performed using a JEOL model JSM-35CF (JEOL Ltd., Tokyo, Japan). Small pieces of aerial mycelium were fixed with 2.5 % glutaraldehyde and dehydrated with alcohol before gold coating and viewing with the SEM.

Chemical Characteristics:

Bacterial cultures were grown for 4-5 days in a Glucose-Yeast Extract medium containing 10 g D-glucose, 10 g yeast extract, 15 g agar, 1 l distilled water, pH 6.8. Diaminopimelic acid and sugars of whole cell

Table 1. Methionine production by *N*-acylamino acid racemase producers after 2 days shaking in medium containing 0.25% glucose, 2.0% peptone, 0.5% NaCl, 0.25% K_2HPO_4 and 0.5% *N*-acetyl-D-methionine.

Strains	L-Met (mM)	D-Met(mM)
Sal21-18	0.14	1.31
Sal21-23	0.12	1.71
Sal25-26	0.15	0.99
Sal25-30	0.09	0.13
Sal30-14	0.04	0.92
Sal30-15	0.07	0.64
Sal30-18	0.17	0.82
Sal31-15	0.21	5.79
Sal31-16	0.07	0.93
Sal34-9	0.08	0.52
Sal34-40	0.11	0.54
Sal35-16	0.68	2.63

hydrolysates were determined according to the methods described by Lechevalier & Lechevalier [5].

Screening for N-acylamino Acid Racemase Producers:

The method described by Tokuyama *et al*[1] was used to screen for *N*-acylamino acid racemase activity. Briefly, actinomycete mycelia of 2-5 day cultures were collected by centrifugation at 4000 g. The reaction mixture was composed of 50 µmol of potassium phosphate buffer (pH 7.0), 25 µmol of *N*-acetyl-D-methionine and the washed mycelia in a total volume of 1 ml. The reaction was kept at 40°C for 20 h, after which the mycelia were removed by centrifugation. One unit of L-aminoacylase and 1 µmol of $CoCl_2$ were added to the supernatant and the reaction was allowed to proceed at 40 °C for another 2 h. The reaction was terminated after 3 min in boiling water. Methionine was detected in the reaction mixture by thin layer chromatography (TLC). The quantity of L-methionine in the reaction mixture was analyzed by high pressure liquid chromatography (HPLC) and strains having L-methionine were selected for analysis in the second screening.

In the second screening, the selected actinomycete strains were cultured again and the mycelia separated into three portions. D-methionine (25 µmol) and *N*-acetyl-D-methionine (25 µmol) were added to the first and second portions but none to the third, which was taken as a control. The reaction proceeded under the same conditions as the first screening. When the reaction was complete, the quantities of L- and D-methionine in the reaction mixture were assayed by HPLC. Strains that produced L-methionine when *N*-acetyl-D-methionine was added were *N*-acylamino acid racemase producers.

The strains that produced an interesting amount of L-methionine in the reaction mixture were identified to species on the basis of the base sequence of the 16 S ribosomal gene.

DNA manipulations:

Primers used in 16S rRNA gene sequencing were as follows:

Primers for PCR technique		Position
9F	5' -GAGTTTGATCCTGGCTCAG	9-27
1510R	5' -GGCTACCTTGTTACGA	1510-1495

Primers for 16S DNA sequencing		Position
9F	5' -GAGTTTGATCCTGGCTCAG	9-27
339F	5' –CTCCTACGGGAGGCAGCAG	339-357
785F	5'-GGATTAGATACCCTGGTAGTC	785-805
1099F	5' –GCAACGAGCGCAACCC	1099-1114

PCR amplification and 16S rDNA sequencing:

Amplification reactions were performed as described in the HotStar *Taq* Master Mix Kit protocol (QIAGEN KK, Tokyo, Japan) in a final volume of 50 µl, containing 10µl of 10x PCR buffer, 25 mM $MgCl_2$, 2µl dNTP (10 mM each), 2µl each 9F and 1510R primers, 5µl template DNA, 0.5µl HotStar *Taq* DNA polymerase and distilled water made up to 50 µl. Chromosomal DNA for PCR template was prepared from cells after culture in trypticase soy broth (TSB) for 1-2 days with the Insta Gene kit (Bio-Rad Corp, Hercules, CA, USA) according to the supplier's protocol. Direct sequencing of purified PCR products was carried out with a Hitachi auto-sequencer SQ-5500S for 10 h at 49° C and 4 primers: 9F, 399F, 785F and 1099F were used for DNA sequencing. After 10 h electrophoresis, all sequences were combined and analyzed to find the most exact match in the GenBank nr database with a BLASTn searching.[6]

Results and Discussion

From 64 sample soils collected from the shore of the Gulf of Thailand in Chonburi, Rayong and Trat Provinces, 567 purified isolates of thermotolerant actinomycetes were obtained and analysed for their capabilities to produce *N*-acylamino acid racemase. Of these, 12 strains appeared to possess the potential to produce *N*-acylamino acid racemase. Strain Sal35-16 was the best *N*-acylamino acid racemase producer among the strains, producing the highest concentration of L-methionine at 0.68 mM (Table 1). The next best producer was strain Sal31-15, which produced L-methionine at 0.21 mM. It also produced 5.79 mM D-methionine in the reaction mixture. All strains with the enzyme activities produced both D- and L- methionine (Table 1).

Both strains grew well on Glucose-asparagine agar,

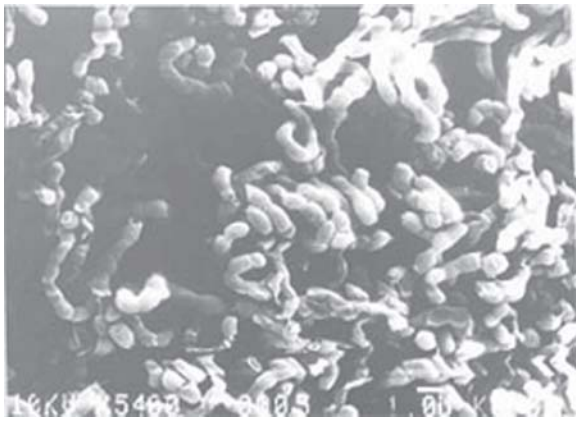

Fig 1. Warty spores of 5 day old strain Sal 31-15 on Starch Casein agar. Scanning electron microscopy, 5400x.

Glycerol- asparagine agar, Yeast-malt extract agar, Oat-meal agar and Starch-casein agar and produced mainly gray spore mass with yellow brown substrate mycelium (Table 2).

Morphological study showed that both strain Sal 31-15 and strain Sal 35-16 produced warty spores in long spiral chains (Fig 1 and Fig 2). Spore and hyphae were similar in diameter and substrate mycelia were non-fragmented.

On the basis of the wall chemotype I, both strain Sal 35-16 and strain Sal 31-15 contained L-diaminopimelic acid and glycine in their cell walls with no sugar in the whole-cell hydrolysate. From the base sequence analysis of the 16 S ribosomal DNA gene, strain Sal 35-16 was identified as *Streptomyces tendae* and Sal 31-15 as *Streptomyces maritimus* (GenBank accession no. AY247715 and AY 247716, respectively).

From the results of this study, *Streptomyces tendae* strain Sal 35-16 produced a small amout of *N*-acylamino acid racemase when compared with the data on *Streptomyces* sp. Y-53 in Tokuyama *et al.*[3]. More work is needed to see if the yield can be improved under suitable conditions for the enzyme production. Furthermore, the rest of the strains could potentially be developed into the good enzyme producers, especially *Streptomyces maritimus* strain Sal31-15, which was an interesting D-aminoacylase producer.

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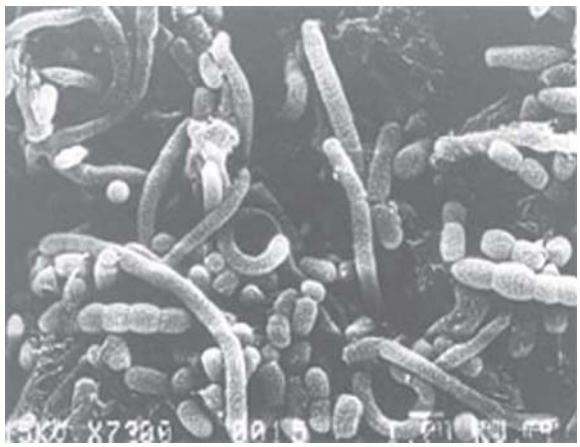

Fig 2. Warty spores of 5 day old strain Sal 35-16 on Starch Casein agar. Scanning electron microscopy, 7300x.

Table 2. Culture characteristics of *Streptomyces tendae* strain Sal 35-16 and *Streptomyces maritimus* strain Sal31-15 on Glucose-asparagine agar, Glycerol- asparagine agar, Yeast-malt extract agar, Oat-meal agar and Starch-casein agar.

Medium	Growth	Arial mycelium	Substrate mycelium
Glucose-asparagine agar			
Sal 35-16	Good	Gray	Yellow-brown
Sal31-15	Good	Yellow-brown	Yellow-brown
Glycerol-asparagine agar			
Sal 35-16	Good	Gray	Dark Yellow-brown
Sal31-15	Good	Gray	Dark Brown
Yeast-malt extract agar			
Sal 35-16	Good	Gray	Yellow-brown
Sal31-15	Good	Gray	Brown
Oat-meal agar			
Sal 35-16	Good	Grayish Black	Yellow-brown
Sal31-15	Good	Grayish Black	Yellow-brown
Starch-casein agar			
Sal 35-16	Good	Gray	Yellow-brown
Sal31-15	Good	Gray	Light Yellow-brown

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