

Characterization of Alkaline Phosphatase Producing Bacteria Isolated from Thai Fermented Fish Products

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Abstract

Seventeen isolates of alkaline phosphatase (ALP) producing bacteria were screened and systematically studied. They were divided into 10 groups on the basis of their phenotypic characteristics and 16S rRNA gene sequence analyses. The Gram-positive coccal isolates in Group I (3 isolates), Group II (3 isolates) and Group III (1 isolate) showed 99.2%, 99.5-99.7% and 99.8% sequences similarity to *Staphylococcus saprophyticus*, *S. napalensis* and *S. sciuri*, respectively. Each Gram-positive rod-shaped isolates in Group IV and V belonged to the genus *Bacillus* and was closely related to *B. vietnamensis* and *B. safensis* with 99.2% and 99.4% sequences similarity, respectively. The Gram-positive, moderately halophilic rod-shaped bacteria in Group VI (3 isolates), Group VII (1 isolate), Group VIII (1 isolate) and Group IX (2 isolates) were closely related to *Virgibacillus halodenitrificans* (98.5-99.1%), *Oceanobacillus iheyensis* (99.3%), *Halobacillus mangrove* (97.9%) and *H. dabanensis* (98.5-98.6%), respectively. One Gram-negative isolate of moderately halophilic bacterium (Group X) was closely related to *Idiomarina zobelli* (98.0%). They possessed phosphatase activities ranged from 10.08-70.96 U ml⁻¹. The isolate NSW 13-2 (Group VI) showed the highest ALP.

Keywords: alkaline phosphatase, fermented fish, *Bacillus*, *Halobacillus*, *Idiomarina*, *Oceanobacillus*, *Staphylococcus*, *Virgibacillus*

1. Introduction

Alkaline phosphatase (ALP) is involved in removing of 5'- and 3'-phosphate groups from DNA, RNA and nucleotides. It can also remove phosphate groups from proteins. This enzyme can be widely found in human tissues, calf, shrimp as well as bacteria. Besides its effect on human health problem, ALP are usefully applied in various fields such as molecular research tool as well as enzyme immunoassays especially bacterial alkaline phosphatase (Dong & Zeikus, 1997; Sun et al., 2007).), milk industry for validation of milk pasteurization (Rankin et al., 2010), cosmetics as a substance for the regeneration and metabolism of cells, environment for monitoring herbicides or pesticides (Muginova et al., 2007), clinical diagnosis as a marker and medicine such as calf intestinal alkaline phosphates, a therapeutic drug for lipopolysaccharide-mediated diseases (Beumer et al., 2003). Despite its wide application, ALP with greater activity or novel properties suitable for commercial applications is still required such as ALP which is active under the high salt concentration and high thermal stability or storage stability.

Halophilic bacteria play important role in Thai traditional fermented fish (*pla-ra*) (Chamroensaksri et al., 2008). In the course of our study on biodiversity of halophilic bacteria in fermented foods, these bacteria are isolated and should be the promising source of bacterial ALP with interesting properties. This work deals with the screening and characterization the halophilic bacteria isolated from Thai fermented fish and salted fish samples that able to produce ALP.

2. Materials and Methods

2.1 Source of Samples and Isolation of Bacteria

Thirty-two fermented fish and salted fish samples were collected from local markets in various provinces of Thailand including Kalasin (KL), Mahasarakham (MK), Rayong (RY), Nakornsawan (NSW), Sukho Thai (SK) and Samut Songkhram (SMK). Isolation of halophilic bacteria from these samples were done by spread plate technique on JCM no. 377 medium composing of 0.5% casamino acids, 0.5% yeast extract, 0.1% sodium glutamate, 0.3% trisodium citrate, 2.0% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2% KCl, 10% NaCl, 0.0036 % $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.00036 % $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.0% agar, pH 7.0-7.2, and incubated at 37 °C for 3-7 days. The colonies on agar plate were selected and purified. The pure cultures were maintained both in lyophilized form and on agar slant kept at 4°C for further study.

2.2 Screening of Alkaline Phosphatase (ALP) Producing Bacteria

The isolates were screened by cultivating on Heart Infusion agar (Difco) containing 0.01 % phenolphthalein bisphosphate tetrasodium salt (Sigma) and 10% NaCl (w/v) as the method described by Barber & Kuper (1951). After incubation at 37 °C for 1-2 days, all pink colonies were selected as potential ALP-producing strains. All selected isolates were separately confirmed for their abilities to produce ALP with the following procedure; a loopfull of the selected ALP-producing strain was inoculated into 5 ml of JCM no.377 broth and incubated on a rotary shaker at 37°C (150 rpm) for 24 h for the seed culture. The seed culture broth (0.5 ml) was transferred into 50 ml of modified JCM No. 377 broth in 250 ml Erlenmeyer flask (duplicate) and incubated as above conditions. Supernatant obtained after centrifugation of the cultures at 10,000 rpm (13,300 g), 4°C for 10 min was used as crude enzyme for ALP activity detection.

ALP activity assay was done by method described by Helianti et al. (2007). Reaction mixture composing of 1.0 ml of 10 mM *p*-nitrophenylphosphate (*p*NPP) (Sigma) in 0.2 M Tris-HCl buffer pH 10.0 with 5 mM MgCl_2 , and 0.1 ml of the crude enzyme was incubated at 37°C for 15 min. The reaction was stopped with 1 ml of 1M NaOH and its absorbance was measured at 405 nm. One unit of alkaline phosphatase (ALP) was defined as the amount of the enzyme yielding 1 micromole of *p*-nitrophenol within 1 minute per milligram protein under the assay conditions. The protein concentration was estimated by Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard.

2.3 Identification Methods

2.3.1 Phenotypic and Chemotaxonomic Characteristics

The colonies grown on JCM no.377 agar in log phase were examined for their cell shape, colonial appearance, pigmentation and Gram staining. Catalase, oxidase, nitrate reduction, MR-VP (Methyl Red-Voges-Proskauer), indole, citrate utilization, hydrolysis of L-arginine, aesculin, gelatin, starch, L-tyrosine and tween 80 were performed as described previously (Thornley, 1960; Barrow & Feltham, 1993). Acid production from carbohydrates was determined in the medium described by Leifson (1963). The biochemical characteristics were recorded after 2 days incubation. Growth at various pHs (4, 5, 6, 7, 8, 9 and 10), NaCl concentrations (0, 3, 5, 10, 15, 20 and 25%) and temperatures (25, 30, 37, 45, 50 and 55 °C) were tested. All tests incubated at 37°C, except for the investigation of the effect of temperatures. Determination of diaminopimelic acid (DAP) in the peptidoglycan was determined as described previously (Komagata & Suzuki, 1987).

2.3.2 16S rRNA Gene Sequence and Phylogenetic Analysis

The 16S rDNA fragments were amplified with 357R, 802R, BF1 and BR1 primers. After purification the PCR products were sequenced using BigDye Terminator Cycle Sequencing Ready Reaction kit (ver. 3.0: Applied Biosystems) in the ABI PRISM 310 Genetic analyzer (Applied Biosystems, USA) The 16S rRNA gene sequence were subsequently aligned along with the selected sequences obtained from the GenBank/EMBL/DBJ databases by using program CLUSTAL X (version 1.81) (Thompson et al., 1997). Gaps and ambiguous bases were eliminated from the calculations. The phylogenetic tree was constructed by using the neighbor-joining method (Saitou & Nei, 1987) in the MEGA program version 4 (Tamura et al., 2007). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1,000 re-samplings.

3. Results and Discussion

3.1 Isolation and Screening of ALP-producing Halophilic Bacteria

Seventy-two isolates of bacteria were isolated from traditional fermented fish and salted fish samples. Of these isolates, only 17 isolates showed pink color on the tested medium indicating that they could produce ALP. These

ALP producing isolates had different ALP activities as shown in Table 1. The isolate NSW 13-2 showed the highest ALP activity (70.96 U ml⁻¹) followed by isolate KL1-1 (49.71 U ml⁻¹), RY30-1 (26.16 U ml⁻¹), RY30-3 (26.05 U ml⁻¹), RY31-2 (25.58 U ml⁻¹), KL2-4 (25.39 U ml⁻¹) and the remained were 10.08-15.12 U ml⁻¹ respectively. The isolate NSW13-2 was provided as a potential ALP-producing strain and capable of growing in the pH range of 7-8 with maximum growth at pH 7.0 that will be useful for further study. Several ALPs have been investigated from the strains of bacteria and fungi such as the hyperthermophilic archaeon *Pyrococcus abyssi* (Zappa et al., 2001); the thermophile *Thermotoga neapolitana* and *Thermus thermophilus* (Pantazaki et al., 1998), the hyperthermophilic archaea, *Aeropyrum pernix* (Helianti et al., 2007), *Bacillus sphaericus* (Dhaked et al., 2005), *Bacillus* sp. (Mahesh et al., 2010) and *E.coli* EFRL 13 (Qureshi et al., 2010), *Bacillus licheniformis* MTCC1483 (Pandey & Banik, 2011), *Rhizopus* *icrospores* var. *rhizopodiformis* (Junior et al., 2008).

Table 1. Isolate number, source, location, nearest relatives, sequence identity (%) and ALP activity of isolates

Isolate no	Product /Province	Nearest relatives	Identity (%)	ALPActivity (U/ml)
Group I <i>Staphylococcus</i>				
NSW25-1	<i>Pla-ra</i> / Nakornsawan	<i>S. saprophyticus</i> subsp. <i>saprophyticus</i> ATCC 15305 ^T	99.3	12.52±0.03
SK22-1	<i>Pla-ra</i> /Sukho Thai	<i>S. saprophyticus</i> subsp. <i>saprophyticus</i> ATCC 15305 ^T	99.2	12.79±0.07
SK23-2	<i>Pla-som</i> /Sukho Thai	<i>S. saprophyticus</i> subsp. <i>bovis</i> GTC 843 ^T	99.2	15.12±0.11
Group II <i>Staphylococcus</i>				
RY30-1	Salted fish /Rayong	<i>S. napalensis</i> CW1 ^T	99.6	26.16±0.01
RY30-3	Salted fish /Rayong	<i>S. napalensis</i> CW1 ^T	99.5	26.05±0.01
RY31-2	Salted fish /Rayong	<i>S. napalensis</i> CW1 ^T	99.7	25.58±0.02
Group III <i>Staphylococcus</i>				
KL6-2	<i>Pla-ra</i> / Kalasin	<i>S. sciuri</i> subsp. <i>sciuri</i> DSM2 0345 ^T	99.8	10.78±0.1
Group IV <i>Bacillus</i>				
KL2-3	<i>Pla-ra</i> / Kalasin	<i>B. vietnamensis</i> 15-1 ^T	99.2	10.08±0
Group V <i>Bacillus</i>				
MK10-2	<i>Pla-ra</i> / Mahasarakham	<i>B. safensis</i> FO-036 ^T	99.4	12.29±0.02
Group VI <i>Virgibacillus</i>				
KL1-1	<i>Pla-ra</i> / Kalasin	<i>V. halodentificans</i> DSM 10037 ^T	99.1	49.71±0.02
KL2-4	<i>Pla-ra</i> / Kalasin	<i>V. halodentificans</i> DSM 10037 ^T	98.6	25.39±0.01
NSW13-2	<i>Pla-ra</i> / Nakornsawan	<i>V. halodentificans</i> DSM 10037 ^T	99.1	70.96±0.01
Group VII <i>Oceanobacillus</i>				
NSW13-4	<i>Pla-ra</i> / Nakornsawan	<i>O. iheyensis</i> JCM 11309 ^T	99.3	13.14±0.06
Group VIII <i>Halobacillus</i>				
NSW13-3	<i>Pla-ra</i> / Nakornsawan	<i>H. mangrove</i> MS10 ^T	97.9	13.22±0.04
Group IX <i>Halobacillus</i>				
NSW13-5	<i>Pla-ra</i> / Nakornsawan	<i>H. dabanensis</i> JCM 12772 ^T	98.6	12.13±0.1
SMK24-1	Hoi-dong/SamutSongkhram	<i>H. dabanensis</i> JCM 12772 ^T	98.5	14.77±0.04
Group X <i>Idiomarina</i>				
MK10-1	<i>Pla-ra</i> / Mahasarakham	<i>Idiomarina zobelli</i> KMM231 ^T	98.0	12.09±0.09

Pla-ra, fermented fish; *pla-som*, fermented fish; *hoi-dong*, fermented molluscs. One unit of ALP was defined as the amount of the enzyme yielding one micromole of *p*-nitrophenol within one minute per milligram protein under the assay conditions.

3.2 Identification and Characterization of ALP-producing Isolates

Seventeen isolates of ALP-producing bacteria were divided into 10 groups based on their 16S rRNA gene sequence analyses and phenotypic characteristics. Seven isolates (Group I, II and III) belonged to the genus *Staphylococcus* were Gram-positive non-motile and non-spore forming cocci. They were catalase-positive but oxidase-negative. Two isolates in Group IV and V belonged to the genus *Bacillus* were Gram-positive spore forming rods. The moderately halophilic bacteria in Group VI (3 isolates belonged to the genus *Virgibacillus*), Group VII (1 isolate belonged to the genus *Oceanobacillus*), Group VIII (1 isolate) and Group IX (2 isolates) belonged to the genus *Halobacillus*, were Gram-positive, spore forming rods. They produced catalase and most of them were oxidase negative. All isolates contained *meso*-diaminopimelic acid in the cell wall. They showed the difference in the colonial pigmentation and the growth in high concentration of NaCl (w/v). One isolate of moderately halophilic bacterium in Group X belonged to genus *Idiomarina* was a Gram-negative non-spore forming rod shaped bacterium. All the isolates in each group were negative reaction for citrate utilization, indole and tyrosine hydrolysis. The details of their characterization are described below.

Group I contained three isolates, NSW25-1, SK22-1 and SK23-2. Colonies were circular, low convex, glistening surface, and gray white with yellowish (4-5 mm in diameter). They grew under aerobic and anaerobic condition in 0-15% NaCl (w/v) for SK22-1 and SK23-2, and 0-20% NaCl (w/v) for NSW25-1 and at 25-45°C (optimum at 30-37°C), at variable optimum pH as shown in Table 2. They were positive for MR but negative for nitrate reduction, hydrolysis of aesculin and starch. They produced acid from D-fructose, D-glucose, glycerol, maltose, mannitol and trehalose, but did not produce acid from L-arabinose, D-cellobiose, D-raffinose, galactose, melibiose, rhamnose, salicin and sorbitol. Isolates SK22-1 and SK23-2 were different from NSW25-1 in acid acid production from sucrose. The other variable characteristics were listed in Table 2. On the basis of 16S rRNA gene sequence analyses (Figure 1), these three isolates were placed in the genus *Staphylococcus*. The isolates NSW25-1 (1459 bp) and SK22-1 (1452 bp) were closely related to *S. saprophyticus* subsp. *saprophyticus* ATCC15305^T with 99.1-99.3% similarity (Trülsch et al., 2007) and the isolate SK23-2 (1460 bp) was closely related to *S. saprophyticus* subsp. *bovis* GTC843^T with 99.2% similarity (Hajek et al., 1996). These three isolates could tolerate to 15% of NaCl as previous reported (Schleifer & Kloos, 1975; Hajek et al., 1996; Trülsch et al., 2007). Therefore, they were identified as *S. saprophyticus* (Schleifer & Kloos, 1975).

Table 2. Differential phenotypic characteristics of *Staphylococcus* isolates and related type strains

Characteristics	Group I			S1	S2	Group II			S3	Group III	
	NSW25-1	SK22-1	SK23-2			RY30-1	RY30-3	RY31-2		KL6-2	S4
Colony colour	gwy	gwy	gwy	gw	cpo	gwy	gwy	w	ow	gwy	gwy
NaCl range (% w/v)	0-20	0-15	0-15	0-15	0-12.5	0-20	0-20	0-20	0-10	0-15	0-10
Temperature range (°C)	25-45	25-45	25-45	15-45	10-40	25-45	25-45	25-45	20-40 (30)	25-45	25-35
Optimum pH	7-8	6-7	7	nd	nd	7	7	7-8	nd	7-8	nd
Alkaline phosphatase	+	+	+	-	-	+	+	+	+	+	+
Voges-Proskauer	+	-	+	+	nd	-	-	-	nd	-	nd
Nitrate reduction	-	-	-	-	+	+	+	-	+	+	+
Aesculin hydrolysis	-	-	-	-	+	+	+	-	+	+	+
Gelatin hydrolysis	+	-	-	nd	nd	-	-	-	nd	+	+
Acid from:											
L-Arabinose	-	-	-	-	-	-	+	+	+	-	d
D-Cellobiose	-	-	-	-	-	-	-	-	-	-	+
D-Fructose	+	+	w	+	+	+	-	-	+	-	+
Galactose	-	-	-	-	+	-	w	-	+	-	+
D-Glucose	+	+	+	+	+	-	w	+	+	-	+
Glycerol	+	+	w	+	+	-	-	-	+	-	+
Lactose	-	-	w	+	-	-	-	w	+	-	-
Maltose	+	+	w	+	+	-	-	w	+	-	+
Mannitol	+	+	w	+	+	-	-	-	+	-	+
D-Mannose	w	-	w	-	-	-	-	+	+	+	d
Melibiose	-	-	-	-	nd	-	-	-	-	-	nd
D-Raffinose	-	-	-	-	-	-	-	-	-	w	+
Rhamnose	-	-	-	nd	+	-	-	-	-	-	d
D-Ribose	w	w	w	-	+	-	-	-	-	+	+
Salicin	-	-	-	-	-	+	+	-	+	+	+
Sorbitol	-	-	-	-	nd	-	-	-	nd	-	+
Sucrose	-	+	+	+	+	-	+	w	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	w	-	-	-	-	-	+	-	+	-	-

S1, *S. saprophyticus* subsp. *saprophyticus* ATCC 15305^T (Schleifer & Kloos, 1975; Trülsch et al., 2007); S2, *S. saprophyticus* subsp. *bovis* GTC843^T (Hajek et al., 1996); S3, *S. napaensis* CWIT (Sperger et al., 2003); S4, *S. sciuri* subsp. *sciuri* DSM 20345^T (Kloos et al., 1976); gw, gray white; cpo, creamy to pale orange; w, white; ow, opaque white; gwy, gray white with yellowish; +, positive; -, negative; w, weak reaction; d, variable; nd, no data.

Group II contained three isolates, RY30-1, RY30-3 and RY31-2. Colonies were circular, low convex, glistening surface, gray white with yellowish (RY30-1, RY30-3), white (RY31-2) and formed 2-5 mm in diameter after 2 days incubation. Growth occurred aerobically and anaerobically in the presence of 0-20% NaCl (w/v), at 25-45°C (optimum at 30-37°C), at optimum pH 7 (RY30-1, RY30-3) and at pH 7-8 for strain RY31-2. They grew well in 10% NaCl (w/v). They were positive for MR and nitrate reduction, but negative for VP and gelatin and starch hydrolysis. The isolate RY30-1 produced acid from D-fructose, salicin and trehalose. The isolate RY30-3 produced acid from D-xylose, D-glucose, galactose, L-arabinose, sucrose and trehalose while the isolate RY31-2 produced acid from L-arabinose, D-glucose, D-mannose and trehalose. On the basis of 16S rRNA gene sequence analyses (Figure 1), these three isolates were placed in the genus *Staphylococcus*. The isolates RY30-1 (1454bp), RY30-3 (1457 bp) and RY31-2 (1456 bp) were closely related to *S. napalensis* CW1^T with 99.6, 99.5 and 99.7% sequence similarity respectively. The ability to produce acid from sugars of these three isolates was variable and was different from *S. napalensis* CW1^T (Spergser et al., 2003) as shown in Table 2. However, they were identified as *S. napalensis* (Spergser et al., 2003).

Group III contained one isolate, KL6-2. Colonies were circular, low convex, glistening surface and gray white with yellowish (less than 3 mm in diameter). Growth occurred aerobically and anaerobically in the presence of 0-15% NaCl (w/v), at 25-45°C (optimum at 30-37°C), at optimum pH 7-8. The isolate was positive for MR, nitrate reduction and hydrolysis of aesculin and gelatin but negative for VP and starch hydrolysis. Acid was produced from D-mannose, D-raffinose, D-ribose, salicin, sucrose and trehalose, but was not from many sugars. The phenotypic characteristics were different from *S. sciuri* subsp. *sciuri* DSM 20345^T (Kloss et al., 1976) as shown in Table 2. The isolate KL6-2 showed 99.8% sequence (1457 bp) similarity to *S. sciuri* subsp. *sciuri* DSM 20345^T based on 16S rRNA gene (Figure 1). Therefore, it was identified as *S. sciuri* (Kloss et al., 1976).

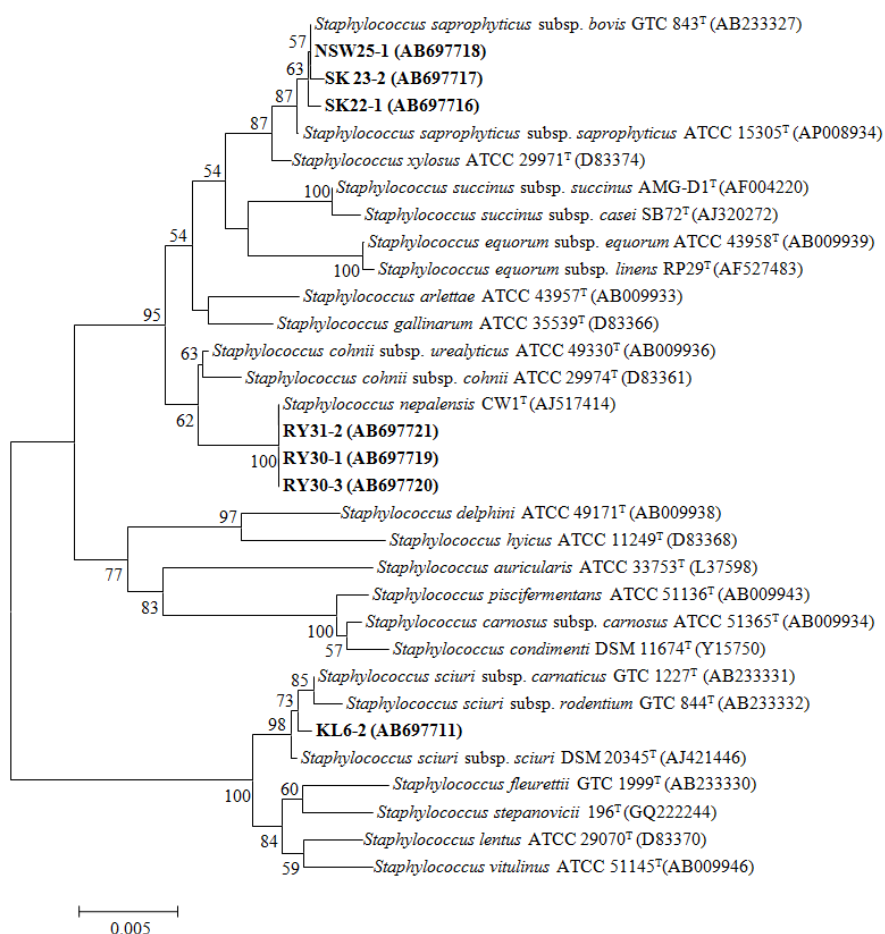


Figure 1. Phylogenetic relationships of isolates NSW25-1, SK23-2, SK22-1, RY31-2, RY30-1, RY30-3, KL6-2 and related taxa based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at nodes. Bar, 0.005 substitutions per 100 nt

Group IV contained one isolate, KL2-3. Colonies were irregular, raised and orange to peach (0.5-3.5 mm diameter). Growth occurred under aerobic and anaerobic conditions, in 0-15% NaCl (w/v), at 25-45°C (optimum at 30-37°C), at pH 7-8. This isolate was positive for hydrolysis of aesculin, gelatin, Tween 80 and starch but negative for MR-VP, arginine hydrolysis and nitrate reduction. Acid was produced from D-fructose, D-glucose, D-ribose, D-trehalose, sucrose, glycerol and maltose. No acid was produced from L-arabinose, D-cellobiose, D-galactose, inositol, inulin, lactose, D-mannitol, D-mannose, D-melezitose, D-melibiose, raffinose, rhamnose, salicin and D-xylose. On the basis of 16S rRNA gene sequence analyses, isolate KL2-3 was placed in the genus *Bacillus* and showed 99.2% sequence (1458 bp) similarity to *B. vietnamensis* 15-1^T (Figure 2). The isolate KL 2-3 showed almost the same phenotypic characteristics as *B. vietnamensis* 15-1^T as shown in Table 3. Therefore, it was identified as *B. vietnamensis* (Noguchi et al., 2004).

Table 3. Differential phenotypic characteristics of *Bacillus*, *Oceanobacillus*, *Virgibacillus*, *Halobacillus* and *Idiomarina* isolates and related type strains

Characteristics	Group IV		Bv	Group V		Bs	Group VI			Vh	Group VII		Oi	Group VIII		Hm	Group IX		Hd	Group X		Iz
	KL2-3			MK10-2			KL1-1	KL2-4	NSW13-2		NSW13-4	NSW13-3		NSW13-5	SMK24-1		MK10-1					
Colony colour	op	co	co	co	dw	cy	cy	cy	c	co	co	py	cb	cy	y	co	y	y				
NaCl range (%w/v)	0-15	0-15	0-15	0-15	0-10	0-20	0-20	0-20	0-23	0-15	0-21	5-20	5-20	1-20	1-20	0.5-25	1-15	1-10				
pH range	7-8	6.5-10	7-8	6.5-10	7-8	6-8	7-8	5.8-9.6	7-9	6.5-10	7-8	6-9	7-8	7-8	5-11	7-8	5.5-9.5					
Temperature range (°C)	25-45	10-40	30-45	10-50	25-45	25-45	25-45	10-45	25-45	15-42	25-45	10-50	25-45	25-45	15-50	25-45	4-30					
Nitrate reduction	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	nd					
Aesculin hydrolysis	+	+	+	+	+	-	-	d	-	nd	-	-	-	-	-	-	-					
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					
Tween 80 hydrolysis	+	nd	-	-	-	-	+	-	-	nd	+	+	+	+	+	+	+					
Starch hydrolysis	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-					
Acid from																						
L-Arabinose	-	-	w	+	-	-	-	-	+	-	-	-	-	-	-	nd	w	-				
D-Cellobiose	-	-	+	+	w	-	w	-	-	-	-	-	+	+	+	nd	+	-				
D-Fructose	+	+	+	+	-	+	+	+	-	-	+	-	+	+	+	+	+	-				
D-Galactose	-	-	+	+	w	w	-	+	+	+	+	+	+	+	+	-	+	-				
D-Glucose	+	+	+	+	w	w	+	+	+	d	+	-	+	+	+	+	+	-				
Glycerol	w	+	w	+	-	-	-	-	+	-	+	+	+	+	+	nd	-	-				
Inositol	-	-	-	+	-	-	-	-	-	+	+	nd	+	+	+	nd	-	-				
Inulin	-	+	-	-	-	-	-	nd	-	-	-	nd	-	+	+	nd	-	-				
Lactose	-	-	-	-	-	-	w	d	-	-	+	+	+	+	+	nd	-	-				
Maltose	w	+	w	+	+	w	w	+	+	d	+	-	+	+	+	+	+	-				
D-Mannitol	-	-	w	+	+	-	w	+	+	+	+	+	+	+	w	+	-	-				
D-Mannose	-	-	+	+	-	-	-	+	+	d	+	-	+	+	-	nd	+	-				
D-Melezitose	-	-	-	-	-	-	-	-	-	-	-	nd	-	-	-	nd	-	-				
D-Melibiose	-	-	-	d	-	-	-	-	-	-	-	-	-	-	-	nd	-	-				
Raffinose	-	-	-	-	-	-	-	-	-	+	+	nd	+	+	+	nd	-	-				
Rhamnose	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	nd	-	-				
D-Ribose	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	+	nd	+	-				
Salicin	-	-	+	+	-	-	-	nd	-	-	+	nd	+	-	+	nd	+	-				
Sucrose	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-				
D-Trehalose	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	-				
D-Xylose	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-				

By, *B. vietnamensis* 15-1^T (Noguchi et al., 2004); Bs, *B. safensis* FO-36^T (Satomi et al., 2006); Oi, *Oceanobacillus iheyensis* JCM 11309^T (Lu et al., 2001); Vh, *V. halodenitrificans* DSM 10037^T (Yoon et al., 2004); Hm, *Halobacillus mangrovei* MS10^T (Soto-Ramírez et al., 2008); Hd, *H. dabanensis* JCM12772^T (Liu et al., 2005); Iz, *Idiomarina zobellii* KMM231^T (Ivanova et al., 2000); c, cream; cb, creamy brown; co, cream orange; op, orange and peach; cw, cream white; cy, cream yellow; dw, dull white; py, pale yellow; y, yellow; +, positive; -, negative; w, weak; d, variable; nd, no data.

Group V contained one isolate, MK10-2. Colonies were round, opaque, creamy white and irregular margins. Isolate KL2-3 was no growth at lower than 30°C. This isolate was positive for MR-VP, hydrolysis of aesculin and gelatin but negative for nitrate reduction, hydrolysis of Tween 80 and starch. Acid was produced from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, D-ribose, salicin, sucrose and D-trehalose. No acid was produced from inositol, inulin, lactose, D-melezitose, D-melibiose, raffinose, rhamnose and D-xylose. The isolate MK10-2 produced no acid from inositol and D-xylose whereas *B. safensis* FO-36^T did (Satomi et al., 2006) as shown in Table 3. This isolate showed 99.4% sequence (1455 bp) similarity to *B. safensis* FO36^T based on 16S rRNA gene sequence analyses (Figure 2). Therefore, it was identified as *B. safensis* (Satomi et al., 2006).

Group VI contained three isolates KL1-1, KL2-4 and NSW 13-2. Colonies were circular to slightly irregular, raised, opaque cream yellow for KL1-1 and NSW 13-2 and cream yellow colour for KL 2-4, (1-2 mm diameter). They grew under aerobic and anaerobic conditions, in NaCl up to 20% (w/v), at 25-45 °C, at pH 7-8 (KL1-1, NSW13-2) and pH 6-8 for KL2-4. They were positive for nitrate reduction and hydrolysis of gelatin but negative for MR-VP, hydrolysis of aesculin and starch. The isolate KL2-4 hydrolyzed Tween 80 but KL1-1 and NSW13-2 did not. Acid was produced from D-glucose, D-ribose, maltose and sucrose. No acid was produced from glycerol, inositol, inulin, D-mannose, D-melezitose, D-melibiose, raffinose, rhamnose, salicin and D-xylose. The variable

of acid production from sugars between these three isolates and the closely type strain was shown in Table 3. The isolates KL1-1(1468 bp), KL2-4 (1477 bp) and NSW13-2 (1474 bp) were closely related to *V. halodenitrificans* DSM 10037^T with 99.1, 98.6 and 99.1% sequence similarity, respectively (Figure 3). On the basis of 16S rRNA gene sequence analyses, these three isolates were placed in the genus *Virgibacillus*. Their phenotypic characteristics were closed to *V. halodenitrificans* DSM 10037^T as shown in Table 3. Therefore, they were identified as *V. halodenitrificans* (Yoon et al., 2004).

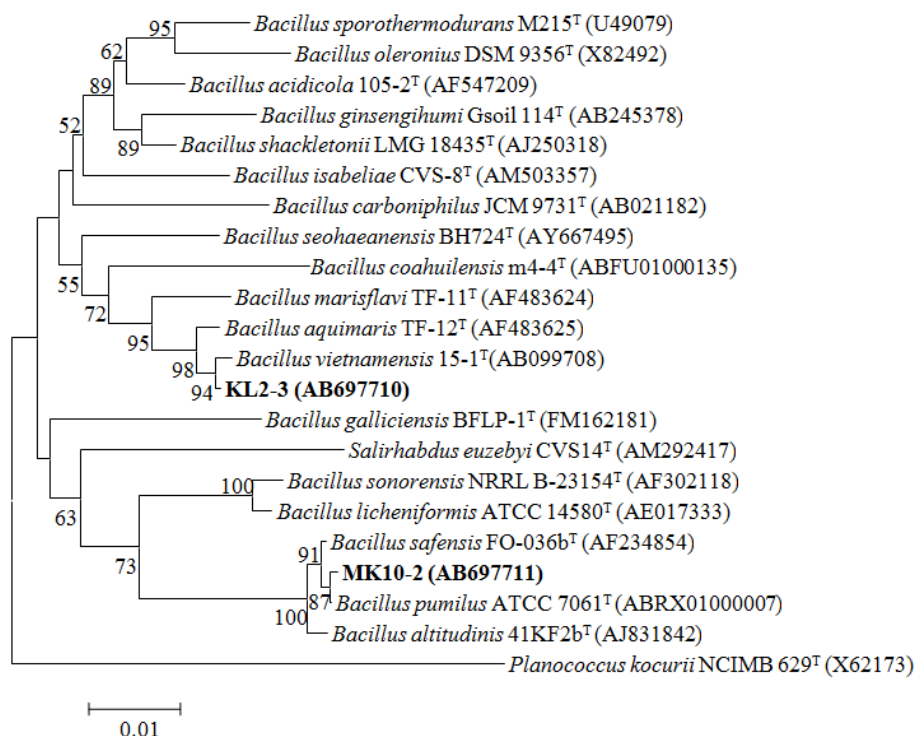


Figure 2. Phylogenetic relationships of isolates KL2-3, MK10-2 and related taxa based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at nodes. Bar, 0.01 substitutions per 100 nt

Group VII contained one isolate, NSW13-4. Colonies were circular, raised and creamy white (2-3 mm in diameter). Growth occurred in the presence of NaCl range 0-15% (w/v), at 25-45°C (optimum at 30-37°C) and at pH 7-9. This isolate was positive for hydrolysis of gelatin but negative for MR-VP, nitrate reduction, hydrolysis of aesculin, arginine, Tween 80 and starch. Acid was produced from L-arabinose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, rhamnose, D-ribose, sucrose and D-xylose. No acid was produced from D-cellobiose, D-fructose, D-galactose, inositol, inulin, lactose, D-melezitose, D-melibiose, raffinose, salicin and D-trehalose. On the basis of 16S rRNA gene sequence analyses, this isolate was placed in the genus *Oceanobacillus*. The isolate NSW13-4 (1467 bp) was closely related to *O. iheyensis* JCM11309^T with 99.3% sequence similarity (Figure 3). The ability to produce acid from sugars of isolate NSW13-4 was variable and was different from *Oceanobacillus iheyensis* JCM 11309^T (Lu et al., 2001) as shown in Table 3. However, this isolate was identified as *O. iheyensis* (Lu et al., 2001).

Group VIII contained one isolate, NSW13-3. Colonies of isolate NSW13-3 were entire, circular, low convex and pale yellow colour. Growth occurred aerobically in the presence of 5-20% (w/v) NaCl, at 25-45°C (optimum at 30-37°C) and at pH 7-8. It was positive for MR, hydrolysis of gelatin, starch and Tween 80 but negative for VP, nitrate reduction and hydrolysis of aesculin and arginine. No acid was produced from L-arabinose, inulin, D-melezitose, D-melibiose, rhamnose and D-xylose. On the basis of 16S rRNA gene sequence analyses, this isolate was placed in the genus *Halobacillus*. The isolate NSW13-3 (1472 bp) was closely related to *H. mangrovi* MS10^T with 97.9% sequence similarity (Figure 3) (Soto-Ramírez et al., 2008). The isolate showed different phenotypic characteristics from *Halobacillus mangrovi* MS10^T (Table 3) and contained *meso*-diaminopimelic acid in the cell wall which was absent in *Halobacillus mangrovi* MS10^T (Soto-Ramírez et al., 2008). Therefore,

this isolate was kept unidentified.

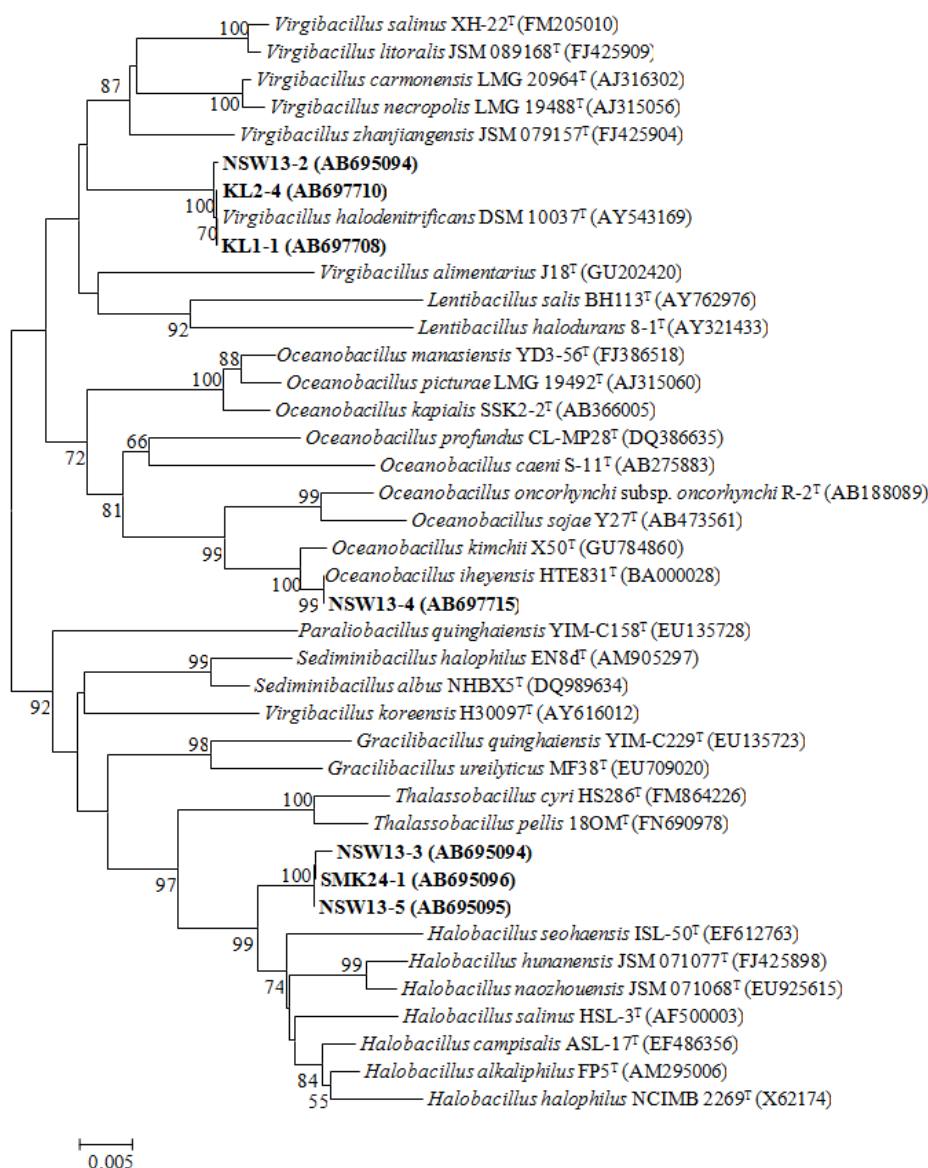


Figure 3. Phylogenetic relationships of isolates KL1-1, KL2-4, NSW13-2, NSW13-3, NSW13-4, NSW13-5, SMK24-1 and related taxa based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at nodes. Bar, 0.005 substitutions per 100 nt

Group IX contained two isolates, NSW13-5 and SMK24-1. Colonies of both isolates were circular, smooth and raised, creamy yellow to yellow (3-4 mm in diameter). Growth occurred aerobically in the presence of 1-20% (w/v) NaCl, at 25-45°C (optimum at 30-37 °C) and at pH 7-8. They were positive for MR, hydrolysis of gelatin, starch and Tween 80 but negative for VP, nitrate reduction and hydrolysis of aesculin and arginine. Both isolates produced acid from D-glucose, glycerol, inositol, maltose, D-mannitol, raffinose, D-ribose, sucrose and D-trehalose. They did not produced acid from L-arabinose, D-melezitose, D-melibiose, rhamnose and D-xylose. The isolate NSW13-5 produced acid from D-cellobiose, D-fructose, D-galactose, lactose, D-mannose and salicin while the isolate SMK24-1 did not as listed in Table 3. On the basis of 16S rRNA gene sequence analyses, both isolates were placed in the genus *Halobacillus*, the isolate NSW13-5 (1467 bp) and SMK24-1 (1468 bp) were closely related to *H. dabanensis* JCM 12772^T with 98.6 and 98.5 % similarity respectively (Figure 3) (Liu et al., 2005). They showed different phenotypic characteristics from *H. dabanensis* JCM 12772^T (Table 3) and

contained *meso*-diaminopimelic acid which was absent in *H. dabanensis* JCM 12772^T (Liu et al., 2005). Therefore, these isolates were kept unidentified.

Group X contained one isolate, MK10-1. Colonies were circular, low convex and light yellowish (2-3 mm in diameter). Growth occurred aerobically in the presence of NaCl range 1-15% (w/v), at temperature range 25-45°C (optimum at 30-37°C) and at pH 7-8. This isolate was positive for gelatin hydrolysis but negative for MR-VP, nitrate reduction, hydrolysis of aesculin, arginine, Tween 80 and starch. Acid was produced from L-arabinose, D-cellobiose, D-glucose, D-mannose, D-ribose, salicin, sucrose and D-trehalose. Whereas the closely type strain *Idiomarina zobellii* KMM231^T did not produce acid from sugars as shown in Table 3. On the basis of 16S rRNA gene sequence analyses, this isolate MK10-1 was placed in the genus *Idiomarina* (Figure 4) and it was closely related to *I. zobellii* KMM231^T 98.0% similarity (Ivanova et al., 2000). The isolate showed different phenotypic characteristics from *I. zobellii* KMM231^T. Therefore, it was kept unidentified.

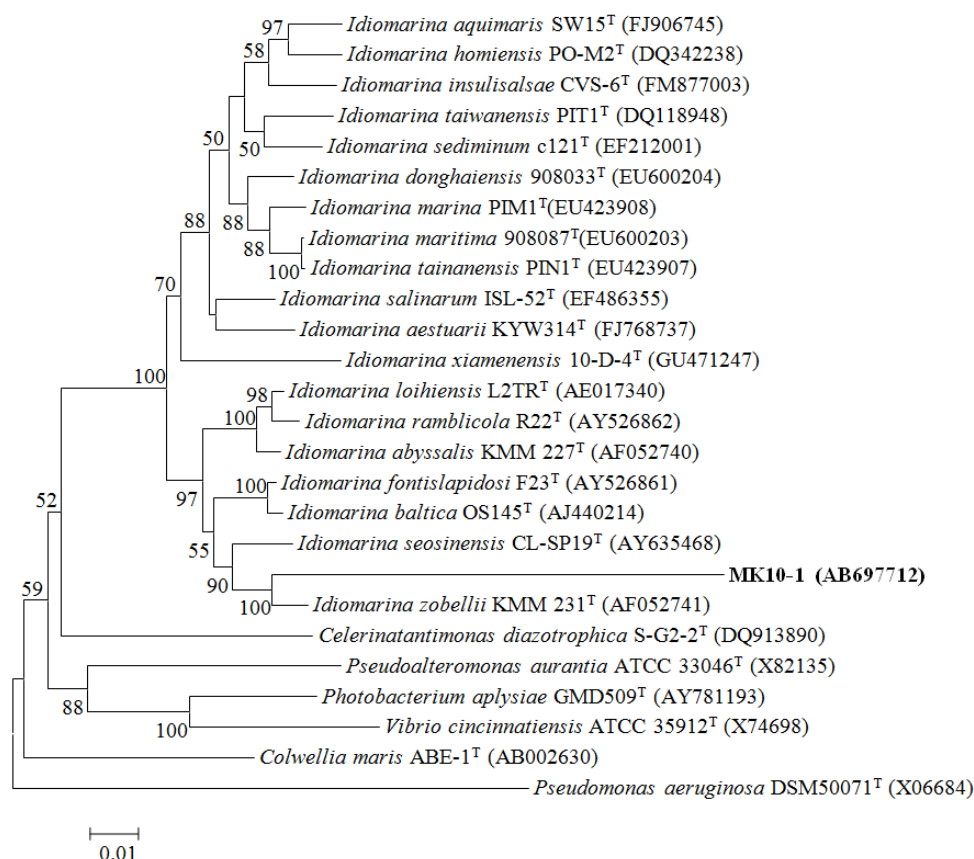


Figure 4. Phylogenetic relationships of isolate MK10-1 and related taxa based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at nodes. Bar, 0.01 substitutions per 100 nt

Recent study, the moderately halophilic strains isolated and identified as *Piscibacillus salipiscarius*, *V. dokdonensis*, *V. halodenitrificans*, *V. marismortui*, *V. siamensis*, *B. vietnamensis*, *Chromohalobacter salexigens*, *Salinivibrio siamensis*, *Gricilibacillus thailandensis* based on 16S rRNA gene sequences and phylogenetic analysis were distributed in fermented fish (*pla-ra*) and salted fishes (Chamroensaksri et al., 2009, 2010; Tanasupawat et al., 2007; 2010). *V. marismortui* strain was characterized as a protease producing moderately halophilic bacterium (Chamroensaksri et al., 2008).

In this study, three isolates in Group I, NSW25-1 and SK23-2 from *pla-ra*, and SK22-1 from *pla-som* identified as *S. saprophyticus* and three isolates in Group II, RY30-1, RY30-3 and RY31-2 identified as *S. napalensis* were isolated from fermented fish with high salt that are different source previous as reported (Schleifer & Kloos, 1975; Spargser et al., 2003). One isolate, KL6-2 (Group III) from *pla-ra* identified as *S. sciuri* was also isolated. One isolate, KL2-3 (Group IV) from *pla-ra* identified as *B. vietnamensis* was found as mentioned above (Tanasupawat et al., 2010). One isolate, MK10-2 (Group V) from *pla-ra* identified as *B. safensis* was found.

Three isolates, KL1-1, KL2-4 and NSW 13-2 (Group VI) from *pla-ra* identified as *V. halodenitrificans* was isolated as mentioned above (Tanasupawat et al., 2010). One isolate, NSW13-4 (Group VII) from *pla-ra* identified as *O. iheyensis* was found. Three novel species of the isolates in Group VIII (NSW13-3 from *pla-ra*) and Group IX (NSW13-5 from *pla-ra* and SMK24-1 from *hoi-dong*, fermented molluscs) were closely related to *H. mangrovi* MS10^T and *H. dabanensis* JCM12772^T, respectively. One isolate, MK10-1 (Group X) from *pla-ra* that related to *I. zobellii* KMM231^T was kept unidentified. The ALP producing bacterial isolates in this study were successfully identified as the genera level, however, the isolates in Group VIII, IX and X are required for further study on their DNA-DNA hybridization to confirm their taxonomic position and to propose them as the novel species.

4. Conclusion

From 32 fermented fish samples, only 17 isolates were shown to be able to produce extracellular ALP. They were identified as the isolates in the genera *Bacillus*, *Halobacillus*, *Idiomarina*, *Oceanobacillus*, *Staphylococcus* and *Virgibacillus* based on their phenotypic characteristics and 16S rRNA gene sequence analysis. The isolate NSW13-2 identified as *V. halodenitrificans* isolated from Nakornsawan fermented fish, was provided as a potential ALP-producing strain. A high level of extracellular alkaline phosphatase production was detected at 70.9 U ml⁻¹. Optimization of ALP production as well as purification and characterization of ALPs from strain NSW13-2 are under study.

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