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Hydrolytic and Inhibitory Activity of Two Closely Related *Bacillus* Isolates

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Abstract Two *Bacillus* strains with different colony morphology were isolated from the soil. Phylogeny and 16S rRNA analysis reveals they both belong to the *Bacillus subtilis* group with both strains showing close relatedness to *B. subtilis* strain 168 and other related strains. These two isolates were examined for cellulase, amylase and lipase activity. Hydrolytic activity was measured via relative enzyme activity. Both strains showed promising results with substrate utilization and *Bacillus sp* strain SI3 displayed a better amylolytic, cellulolytic and lipolytic activity. Proteolytic ability was also assessed using skimmed milk agar and gelatin. Furthermore, the culture extracts of both isolates were examined for antimicrobial activity. This work demonstrates that these strains could have potential application in the industrial production of these economically important enzymes and other bioactive compounds.

Keywords: *Bacillus, cellulase, antimicrobial, amylase, hydrolytic*

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1. Introduction

Bacillus are spore forming bacteria that can withstand unfavorable environmental conditions. They are also known to produce diverse antimicrobial compounds [1]. In addition, they are capable of synthesizing different extracellular enzymes, which empower them to utilize different substrates. Enzymes such as cellulase/endoglucanase, chitinase, lipase, protease etc. have been reported in *Bacillus*, all of which do not only aid the survival or defense of the organism but can also be harnessed for industrial processes such as production of food, textile, leather, biofuels etc. [1,2]. *Bacillus* spores have been used as additives or antimicrobial in poultry feeds, bio-pesticides and growth enhancer for plants [1,3]. Moreover, their ability to survive the conditions in the gut plus the exogenous enzymes they produce enables them to break down complex food substance and improve nutrient availability as feed additives [1,4]. These qualities also prevent the hazards that comes with antibiotics growth promoters or chemical pesticides.

Compared to *Saccharomyces*, another organism of biotechnological significance, *Bacillus sp* grow faster, and thus have a shorter fermentation cycle as well as a great physiology and better survivability in difficult environments [5]. They can also utilize and break down cheap carbon sources for energy, making them of huge interest for industrial applications. Furthermore, they are genetically malleable with different stable expression systems available [5].

This study primarily seeks to analyze some key enzymes produced by two closely related *Bacillus* isolates, their hydrolytic capacities and potential application for industrial use. In addition, the antimicrobial activities of these isolates were also evaluated. Microorganisms such as bacteria and fungi have been studied for the biosynthesis of enzymes with industrial applications. Enzymes from microbes serve as biocatalysts and can drive reactions in an economical and more environmentally friendly manner than chemical catalysts [2]. They are also stable over a wide range of temperature, pH etc. as some microorganism can thrive in extreme environments [6,7].

2. Materials and Methods

2.1. Strain Isolation

Dilutions from a soil sample obtained from Lafayette, Louisiana, were plated on glycerol yeast extract agar (5g/L Glycerol; 2g/L yeast extract, 1g/L K_2HPO_4, 15g/L agar). Two colonies were selected for further studies based on their morphology. Isolates were cultivated and propagated on tryptic soy agar (TSA)(15 g/L tryptone, 5 g/L soytone, 5 g/L sodium chloride, 15g/L agar) [8].

2.2. PCR Amplification and Sequencing

The forward primer; 63f -5'-CAG GCC TAA CAC ATG CAA GTC-3' and reverse primer; 1387r-5'-GGG CGG WGT GTA CAA GGC-3' were used in amplifying

the 16S rRNA genes of isolates via colony PCR [9]. Amplification included an initial denaturation at 95°C for 3 min., followed by 25 cycles consisting of denaturation at 95°C for 30s, primer annealing at 48^0C for 30s, extension at 72°C for 45s and then a final extension step for 5 minutes at 72°C. Sanger sequencing of purified PCR samples was carried out by functional biosciences (WI, USA). The DNA sequences were submitted to the Basic Local Alignment Search Tool (BLAST, NCBI) for analysis. The MEGA software (version 7) was used for phylogeny construction [10].

2.3. Antimicrobial Activity

The TSA plates cultures of isolates were incubated for 5 days. To recover secondary metabolites, an agar extraction method was carried out [11]. The agar culture was cut into small pieces, soaked in methanol and covered with parafilm for 24 hours, after which the methanol extract was recovered and dried in a chemical hood. Dried extracts were re-suspended in 0.5ml methanol and 40µl of the suspension was loaded on sterile filter paper discs. The discs were then placed on TSA plates swabbed with the test organisms. Plates were observed for zone of inhibition after 24-36 hrs.

2.4. Media Used for Hydrolysis Test

M9 minimal media which is composed of 3 g/L KH_2PO_4, 6g/L Na_2HPO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1mM $MgSO_4$, 15g/L agar, and 1ml/L trace element solution (50g/L Na_2-EDTA, 22 g/L $ZnSO_4 \times 7H_2O$, 5.54 g/L $CaCl_2 \times 2H_2O$, 5.06 g/L $MnCl_2 \times 4H_2O$, 5 g/L $FeSO_4 \times 4H_2O$, 1.10 g/L $NH_4Mo_7O_{24} \times 4H_2O$, 1.57 g/L $CuSO_4 \times 5H_2O$, 1.61 g/L $CoCl_2 \times 6H_2O$) was prepared and supplemented with different substrates [8]. Carboxy-methyl cellulose (CMC) (10g/L) was added to the media for cellulase activity testing. For lipase test, 30ml/L difco lipase reagent (tributyrin & polysorbate 80) was added. For amylase test, difco starch agar (3g/L beef extract, 10g/L soluble starch and 15g/L agar) was used. Tryptic soy agar (TSA) with 10% skimmed milk was utilized for casein hydrolysis test. TSA was supplemented with 5% sheep's blood for hemolysis test. Tryptic soy broth (TSB) with 12% gelatin was used for gelatinase test [3,8].

Cellulase was detected via clearance zone by staining 72 hours old CMC agar cultures with 1% congo red for 20 min followed by destaining with 1M NaCl for 10 min. Amylase was detected after staining starch agar cultures with iodine solution. Clearance zone was observed for lipase and casease after cultivating strains on respective media for 72 hours. For gelatinase test, liquefaction was observed after gelatin tubes cultures incubated at 37^0C for 48 hours were refrigerated for 30min.

2.5. Enzyme Activity

The enzyme activity of cellulase, amylase, lipase was measured via hydrolytic capacity or relative enzyme activity (REA) which is the ratio of clear zone diameter to colony diameter [1,12]. A larger ratio was treated as a higher enzyme activity.

2.6. Statistical Method

Mean REA and standard deviation of triplicate tests were calculated and reported as mean ± standard deviation. To determine statistical significance, a student t test was performed.

3. Results and Discussion

Two *Bacillus* strains were isolated from the soil and named *Bacillus sp* strain SI3 and *Bacillus sp* strain SI4. BLAST analysis reveals that both strains are members of the *B. subtilis* group and are closely related to *B. subtilis* strain 168 with SI3 and SI4 showing a 99.59% and 99.86% identity respectively. The *B. subtilis* group includes *Bacillus subtilis* subsp. *subtilis*, subsp. *spizizenii and* subsp. *inaquosorum*, *Bacillus amyloliquefaciens*, *B. licheniformis*, *B. malacitensis*, *B. mojavensis*, *B. pumilus*, *B. sonorensis*, *B. tequilensis*, *B. velezensis* etc. [13].

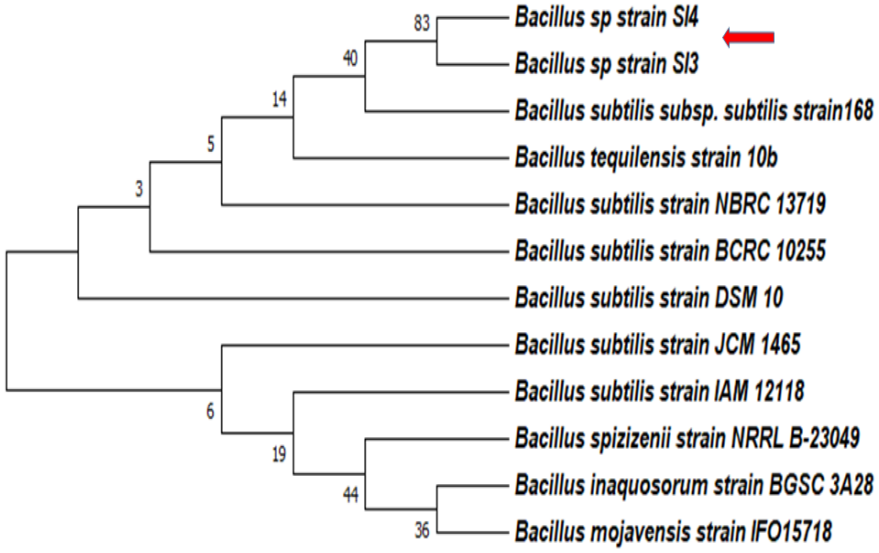

Figure 1. Phylogeny of *Bacillus sp* strain SI3 and *Bacillus sp* strain SI4. Neighbor joining tree was constructed using 16S rRNA sequences. Bootstrap values based on 100 replicates is shown at the nodes

Similarly, phylogenetic analysis suggests that both strain SI3 and SI4 are related to *B. subtilis* strain 168 (Figure 1). They also share the same clade with *B. tequilensis* and some other *B. subtilis* strains (Figure 1). *B. tequilensis* has been reported to share a 99% similarity with *B. subtilis* based on 16S rRNA analysis but has less than 70% homology with *B. subtilis* and other related strains based on DNA-DNA hybridization studies [13]. Both isolates also have unique colony morphology with SI3 producing white, irregular, raised colonies while SI4 produced cream colored, flat, rough, undulate colonies (Figure 2).

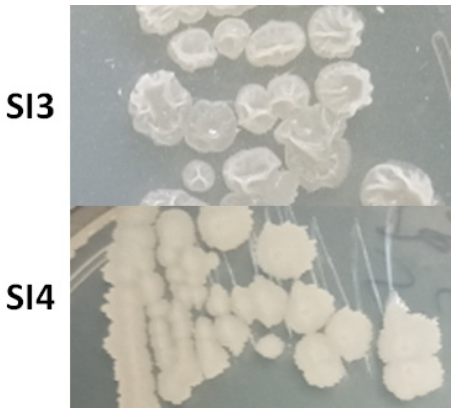

Figure 2. Colony morphology of strain SI3 and SI4

A study investigated the hydrolytic activities of 31 *Bacillus* isolates, calculated the relative enzyme activity (REA) of different enzymes and categorized enzyme activity as poor if REA<2, good if REA is between 2 and 5, and excellent if REA> 5 [1].

Good cellulase activity with REA of 3.6 and 2.3, was observed for both strains indicating their potential as promising industrial cellulase producers (Figure 3A; Table 1). A study reported cellulase activity ranging from 1.3 to 2.1 [12], while another one reported a range of 2-4.5 from *Bacillus* strains [14]. Cellulase would allow for the cheap recovery of glucose from lignocellulose biomass. This could help reduce the production cost of ethanol and biofuels [2]. It is also an essential resource for the paper and pulp industry. Xylanase, which breaks down hemicellulose has also been reported in *B. subtilis* strain 168 [15].

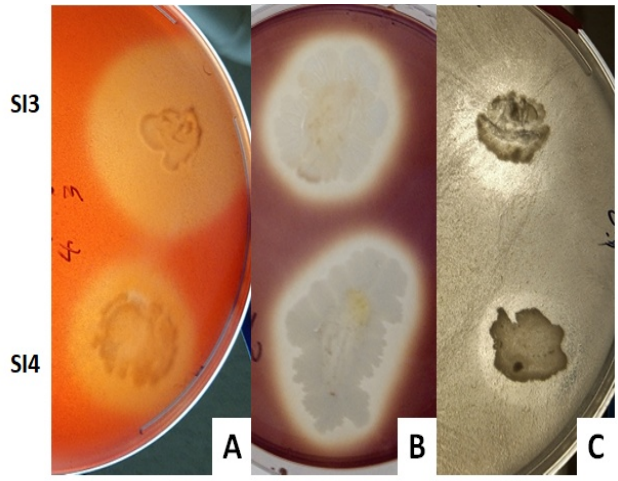

Figure 3. Hydrolysis of cellulose (A), starch (B) and Lipids (C) by strain SI3 (Top) and SI4 (Bottom)

Both strains hydrolyzed starch (Figure 3B). Amylases from isolated *Bacillus* strains with a REA range of 1.8 to 4.22 have been reported [14]. Another study reported a REA range of 1- 6.3 for *Bacillus* isolates [1]. The REA observed for amylase here was lower than that observed for most of the *Bacillus* strains in both studies (Table 1). Amylase is essential for starch processing in the paper industry or industrial food processing such as brewing or high corn fructose syrup production [2]

Table 1. Hydrolytic activities of Strain SI3 and SI4. Different letters within the same column indicate statistical significance (p < 0.05)

Strains	Relative Enzyme Activity (REA) (clear zone diameter/colony diameter)		
	Cellulase	Amylase	Lipase
SI3	3.60 ± 0.36^{a}	1.82 ± 0.06^{a}	2.04 ± 0.24^{a}
SI4	2.28 ± 0.25^{b}	1.73 ± 0.04^{b}	1.86 ± 0.15^{b}

Lipase activity was observed for both strains SI3 and SI4, with a REA of 2 and 1.86 respectively (Figure 3C; Table 1). The REA obtained here is similar to that from another study which reported a range of 1- 3 for lipase [1]. Lipase together with another enzyme such as protease can be added to detergents to improve the durability of textiles. Lipase may also be used to remove hydrophobic or oily component of wood during pulp production. [6]. Both strains displayed proteolytic activity with the hydrolysis of gelatin as well as casein with SI3 producing a bigger clearance zone on milk agar (Table 2). Similarly, both tested positive for catalase (Table 2).

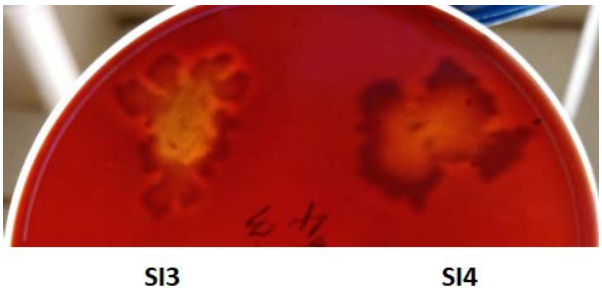

Figure 4. Hemolytic activity of strain SI3 and SI4

Additionally, both strains showed hemolytic activity on blood agar with strain SI3 displaying a stronger activity (Figure 4; Table 2). Hemolysis can be used as a tool for detecting the production of biosurfactants which reduces surface tensions and interfacial tension. This quality promotes their use as cleaning agents as well as in emulsification and biodegradation of petroleum products [16].

Table 2. Biochemical tests and antimicrobial activity of Strain SI3 and SI4. All tests were performed in triplicates

Tests	Strains	
	SI3	**SI4**
Catalase	+	+
Gelatinase	+	+
Hemolysis	+	+
Casein hydrolysis **Avg. Clear zone diameter (mm) ± SD**	25 ± 0.15	13 ± 0.21
Antimicrobial activity **Avg. Zone of inhibition diameter (mm) ± SD** ***M. luteus***	13 ± 0.06	10 ± 0.10

The antimicrobial activity of both strains was evaluated by testing culture extracts against *Micrococcus luteus* and *Escherichia coli*. Both strain SI3 and SI4 produced inhibitory activities against *M. luteus* with inhibition zone diameters of 13mm and 10mm respectively (Table 2). *Bacillus* are known to produce secondary metabolites as well as antimicrobial peptides such as mersacidin which has potency against some gram positive pathogens such as *Staphylococcus aureus* [17]. Some of these peptides are produced via enzyme complexes and are thus non-ribosomally synthesized while the others are ribosomally produced. Increasing cases of antibiotic resistance calls for the investigation and development of new antimicrobial agents [11].

4. Conclusion

This study demonstrates that two closely related *Bacillus* strains isolated from the soil could produce bioactive metabolites and enzymes with the ability to hydrolyze substrates to varying degrees. Strain SI3 displayed higher cellulase, amylase and lipase activity as well as a better proteolytic and hemolytic ability compared to strain SI4. Future work is required on how to maximize enzyme production and recovery from these strains. This may involve genetic modification of the metabolic pathways in these strains via mutagenesis or identifying and overexpressing the putative biosynthetic genes in an ideal host. Culture conditions such as pH and temperature should also be optimized to determine enzyme properties and promote yield. Investigating the nature of the antimicrobial metabolites produced by isolates via chromatography would be ideal. This work further underscores the significance of *Bacillus* as promising industrial enzyme producers and potential use as antimicrobial producers, animal feeds additives, and probiotics to help break down complex substances while promoting healthy gut health [18].

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