Screening of Industrially Important Enzymes Produced by Moderately Halophilic Bacteria Isolated from Salted Sheep Skins of Diverse Origin

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Abstract

Moderately halophilic bacteria have received attention in several industries due to their industrial enzymes which are stable at high temperatures, various salt concentrations and different pH values. Therefore, this study was conducted to isolate and identify moderately halophilic bacteria found on salted sheep skin samples and to detect the isolates producing industrially important enzymes. These skin samples were from Australia, Bulgaria, Dubai, Greece, Israel, Kuwait, South Africa, Turkey and U.S.A. Phenotypic characteristics and comparative partial 16S rRNA gene sequence analysis were used to characterize these microorganisms. According to the test results, 77 isolates representing 13 genera and 29 species were identified. These moderately halophilic bacteria, which were able to mostly grow in the media containing 3-15% salts and in some cases, up to 20-25% salts, were closely related to species of genera *Staphylococcus*, *Salimicrobium*, *Bacillus*, *Salinicoccus*, *Planococcus*, *Alkalibacillus*, *Gracilibacillus*, *Oceanobacillus*, *Marinococcus*, *Halomonas*, *Salinivibrio*, *Chromohalobacter*, and *Idiomarina*. A fairly high percentage of the isolates (79%) produced a great variety of industrially important enzymes. Protease, lipase, β-galactosidase, amylase, caseinase, DNase, urease, cellulase, and lecithinase enzymes were produced respectively by 46, 33, 30, 20, 18, 13, 9, 9, and 8 isolates. None of the isolates produced pullulanase, xylanase and phospholipase enzymes. Combined enzymatic activities have been detected among the isolates. While 12% and 27% of isolates produced six and four different enzymes, respectively, 1% of isolates produced three different enzymes. Furthermore, 39% of the isolates produced one and two enzymes. These enzymes were produced by isolates belonging to all genera detected in this study except genus *Alkalibacillus*. The findings of this study demonstrated that moderately halophilic bacteria on the skins produced industrially important enzymes which may have potential applications in different industries.

Introduction

Enzymes, which are biological catalysts, have a wide variety of commercial applications in different industries. Although enzymes are produced from plants, animals, fungi, bacteria and yeasts, bacterial and fungal enzymes are mostly used for numerous industrial applications owing to amenability to genetic manipulation, inexpensive and easy production.[1-4] Amylase, protease, cellulase, DNase, caseinase, urease, lecithinase, phospholipase, xylanase, lipase, pullulanase and β-galactosidase enzymes have potential applications in food, textile, beverage, baking, paper, pharmaceutical, starch, laundry, meat, dry cleaning, feed and dairy industries.[1,5-7]

Enzymes which may be active at different salt concentrations, pH values and temperatures are usually preferred in various industries.[8] Moderately halophilic aerobic microorganisms, which are able to grow at 3-15% NaCl, 0-45°C and pH values of 5-10, have been thought to be potential source of such salt-adapted enzymes.[5] Hence, enzymes of moderately halophilic bacteria have drawn attention in diverse industries.[7] When halophilic bacteria are used in industrial processes, expenses for sterile conditions are unneccessary.[5] Therefore, using moderately halophilic bacterial enzymes in different industrial applications may reduce processing costs remarkably. Researchers emphasized that moderately halophilic bacteria isolated from various saline environments in Spain were able to produce pullulanase, amylase, protease, xylanase, lipase and DNase enzymes.[8] In another study, moderately halophilic bacteria, isolated from Howz Soltan playa (a hypersaline lake), produced proteases, cellulases, lipases, xylanases, inulinases, pullulanases and amylases.[9] Moreover, moderately halophilic strains were isolated from soil, sea water and water samples which were collected from saline habitats of India. Amylases, lipases and proteases were produced by some of these halophilic isolates.[10]

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Moderate halophiles that exhibit combined hydrolytic activities may offer great advantage for the various industries. Moderately halophilic bacteria producing different enzymes may be used for purification of polymer containing wastes.[8] Halophilic proteases and lipases that work in different salt concentrations, temperatures and pH values may be used in hide brine curing liquors and soak liquors to remove globular proteins and fat from the hides and skins, respectively. These enzymes are also used in depilatory, degreasing and bating processes in leather industry.[11] Halophilic protease may be a candidate in food processing.[12] Lipases and amylases have a wide range of applications involving detergent, chemical, food and pharmaceutical industries.[8] Lipase may have a potential application in dairy industry to hydrolyse milk fat.[12] This enzyme may be used in the treatment of oilfield waste and bioremediation of oil spills in marine environments.[8,12] In addition, lipase enzyme of halophilic *Haloarcula hispanica* 2TK2 was mentioned as a good candidate for detergent and paper manufacturing, biodiesel and leather industries.[13] Galacto-oligosaccharides have been used as prebiotic agents in food industry. β-galactosidase has been used in the synthesis of galacto-oligosaccharides from lactose.[14] Cellulase produced by moderately halophilic bacteria may be used in food and fermentation industries, treatment of agricultural waste, and bioremediation of cellulose material.[12,15] DNase enzymes have potential as a flavoring agent in food industry.[12] Xylanase produced from halophilic *Bacillus* sp. has been used in xylan biodegradation in pulp and paper industries.[2] Pullulanase produced from halophilic *Bacillus* sp. are used in biocatalysis in organic solvents and super critic fluids.[16]

Although enzymes of moderately halophilic bacteria isolated from hypersaline environments such as saline lakes, salterns, sea water and saline soils[8-10] were examined, the enzymes of moderately halophilic bacteria obtained from salted sheep skins have not been investigated in detail. Hence, the goal of this study was to isolate and identify moderately halophilic bacteria that produce industrially important enzymes. Moderately halophilic bacteria were isolated from 23 salted sheep skins obtained from nine countries and these isolates were characterized using phenotypic characteristics and comparative partial 16S rRNA gene sequence analysis. The industrial potential of these isolates was investigated by examining amylase, DNase, cellulase, caseinase, lipase, protease, urease, pullulanase, xylanase, lecithinase, phospholipase and β-galactosidase enzymes. In addition, biochemical activities and production of acids from different carbon sources by the isolates were examined in this study.

Experimental

Salted Sheep Skin Samples

Twenty-three salted sheep skin samples obtained from different countries [Australia (4), Bulgaria (2), Dubai (3), Greece (4), Israel (2), Kuwait (1), South Africa (2), Turkey (2) and U.S.A. (3)] were collected from tanneries in Tuzla and Corlu Leather Industrial Zones, Turkey. Then, the samples were immediately placed into sterile sample bags and transported on ice. The samples were coded according to origin: Australia (ATY, AV, AVS, A); Bulgaria (BL, C); Dubai (DB, DBA, D); Greece (YN, L, IR, Y); Israel (ISR, ISL); Kuwait (KV); South Africa (GAF, GA); U.S.A. (EK, MK, GAM); and Turkey (TRK, TR) (Table I).

Isolation of Moderately Halophilic Bacteria

The skin samples, weighing 20 g, were cut and soaked separately in glass flasks containing 180 mL 10% NaCl solution. The flasks were placed into a shaking incubator at 100 rpm for 4 hours at 25°C. The suspension of the skin was diluted with sterile physiological saline water containing 10% NaCl. An aliquot of 0.1 mL of each, direct and serial dilutions (from 10^{-1} to 10^{-6}) of skin suspension, was spread onto the surface of the agar plates containing Complex Medium I (CMI) supplemented with 0.5% (w/v) yeast extract. The final salt concentration of all test media was 10% (w/v) with the following composition (SW10, saline water): 0.7% (w/v) $MgCl_2$, 0.96% (w/v) $MgSO_4$, 8.1% (w/v) NaCl, 0.2% (w/v) KCl, 0.036% (w/v) $CaCl_2$, 0.0026% (w/v) NaBr and 0.006% (w/v) $NaHCO_3$.[17] The plates were incubated at 37°C for 24 h. Following incubation, different bacterial colonies were selected and restreaked several times to obtain pure cultures, then subjected to phenotypic and genotypic analysis.

Amplification and Sequencing of 16S rRNA Genes of the Isolates

Chromosomal DNA was isolated and purified by QIAamp DNA Mini Kit (Qiagen) and QIAquick PCR Purification Kit (Qiagen). These procedures were conducted according to the manufacturer's instructions. The 16S rRNA genes of the isolates were amplified by PCR using forward and reverse universal primers: 16F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and 16R1488 (5'-CGGTTACCTTGTTAGGACTTCACC-3').[18] The 16S rRNA gene sequences (1038-1477 bp) were determined by IONTEK Laboratory (Turkey). We further determined 16S rRNA gene similarities (98.1-100%) between isolates and closely related species by using ChromasPro and EzTaxon-e tool.[19] Phylogenetic trees were constructed using the neighbor-joining method and MEGA 4.0.[20,21] The gene sequence data in the present study were deposited in GenBank under accession numbers.

Morphological, Cultural and Physiological Characteristics of the Isolates

Exponentially growing isolates were examined for cell morphology and pigmentation. Cell morphology of each isolate was examined on prepared wet mounts using light microscopy. Gram staining was performed by using acetic acid-fixed slides.[22] Salt requirement and salt tolerance of moderately halophilic bacteria were investigated on plates of the above-described medium in which the salt concentration was varied (0, 0.5, 3, 5, 7.5, 10, 12.5, 15, 20 and 25%). The pH tolerance of the isolates was tested in CMI agar medium adjusted to pH values of 4, 5, 6, 7, 8, 9, 10 and 11. To determine optimum growth temperature of the isolates, the plates inoculated with each isolate were incubated at different temperatures (4, 28, 35, 37, 40, 45°C). Catalase and oxidase activities, citrate utilization, indole production, methyl red and Voges-Proskauer tests of each isolate were investigated using earlier described procedures.[23] Triple Sugar Iron Agar was used to determine test isolates' ability to produce H_2S and to ferment glucose, lactose and sucrose. Reduction of nitrate to nitrite was determined in tubes containing nitrate broth medium supplemented with 2% $KN0_3$. The presence of nitrite was detected with naphthylamine/sulphanilic acid reagents, and production of N_2 was observed in Durham tubes. Production of NH_3 from peptone was detected using Nessler's reagent. Formation of brown precipitate in the test medium after addition of the test agent was accepted as a positive test result. The acid production from different carbon sources was separately examined using 1% (w/v) lactose, sucrose, D-glucose, D-galactose, D-trehalose, D-melibiose, D-mannose, D-xylose, D-cellobiose, L-arabinose, fructose, maltose, 0.5% (w/v) yeast extract, and 0.001% (w/v) phenol red.[23]

Determination of Enzymatic Activities of the Isolates

The salt mixture SW10 was added into the following test media. The pH of all media was adjusted to 7.5. Amylase activity of the isolates was determined using CMI agar medium supplemented with 0.5% (w/v) soluble starch. After incubation of test isolates, the plates were flooded with 0.3% I_2-0.6% KI solution. A clear zone around the colonies indicated starch hydrolysis. The DNase test agar was used to determine DNase activity of the test isolates. After incubation, the plates were flooded with 1N HCl. Clear zones around the colonies indicated hydrolysis of DNA.[8] The cellulose medium containing 0.2% (w/v) carboxymethyl cellulose was used to detect production of cellulase by the isolates. After incubation, 0.1% congo red test reagent was flooded on the colonies and left for 30 min. Afterwards, the colonies were washed with 1 M NaCl solution. Clear zones around the colonies were evidence of positive cellulase activity.[24] Hydrolysis of casein was tested with plate count agar media containing 2% skim milk. After incubation, clear zones around the colonies were interpreted as caseinase production.[8] Lipase activity of the isolates was screened on Tween 80 agar medium containing 1% (w/v) Tween 80. After incubation, opaque zones around the colonies were interpreted as evidence of lipase activity.[24] Phospholipase activity of the isolates was also tested in agar medium containing 5% (w/v) butter. After incubation, 20% cupper-sulphate solution was flooded onto the plates. Positive bluish green colonies were interpreted as butter hydrolysis.[25] Proteolytic activity was screened on gelatin agar medium containing 2% gelatin (w/v). After growth was obtained, the plates were flooded with Frazier solution. Clear zones around the colonies indicated protease activity.[8] Urease activity of the test isolates was detected on Christensen urea agar medium. After growth was obtained, the tubes were scrutinized for pink or red color changes.[23] Pullulolytic activity of the isolates was tested in CMI agar medium supplemented with azurine-cross-linked (AZCL)-pullulan. Following the incubation period, pullulolytic activity of the isolates was detected by clear zones around the colonies. Xylanolytic activity of the isolates was tested in CMI agar medium supplemented with AZCL-xylan. After the incubation period, xylanolytic activity of the isolates was detected by clear zones around the colonies.[8] To examine lecithinase activity of the isolates, lecithine agar plate containing 5% egg yolk (w/v) was used. Opaque zones around the colonies were interpreted as evidence of lecithinase activity.[25] β-galactosidase activity of the isolates was detected in test tubes containing 1 mL of sterile distilled water containing 10% NaCl (w/v) and ONPG (*ortho*-nitrophenyl-β-galactoside) discs. The formation of yellow color was accepted as positive β-galactosidase activity.[24]

Results and Discussion

In the present study, forty-one and thirty-six isolates were belonging to phyla *Firmicutes* and *Proteobacteria*, respectively. Gram-positive genera such as *Staphylococcus, Salimicrobium*, *Bacillus*, *Salinicoccus*, *Planococcus*, *Alkalibacillus*, *Gracilibacillus*, *Marinococcus* and *Oceanobacillus*, found in phylum *Firmicutes*, were detected (Table I).

Phylogenetic trees were constructed based on the comparison of 16S rRNA gene sequences of reference strains showing the higher similarities. These trees show the phylogenetic position of our strains isolated from salted sheep skin samples. The pairwise sequence similarities were between 98.4-100% for the isolates belonging to *Firmicutes*, and 98.1-100% for the isolates belonging to *Proteobacteria* (Fig.1a-b).

Eighteen different moderately halophilic bacterial species in phylum *Firmicutes* were obtained from salted skins. Four different Gram-negative genera such as *Halomonas*, *Salinivibrio*, *Chromohalobacter* and *Idiomarina*, found in phylum *Proteobacteria*, were determined. Eleven different species belonging to *Proteobacteria* were identified. While the most abundant species on the skins was *Bacillus licheniformis* (n=7), the least abundant species were *Staphylococcus saprophyticus*

subsp. *saprophyticus*, *Bacillus siamensis*, *Planococcus rifietoensis*, *Alkalibacillus halophilus, Marinococcus luteus*, *Marinococcus tarijensis*, *Oceanobacillus picturae*, *Halomonas eurihalina*, *Chromohalobacter israelensis* and *Idiomarina loihiensis* (n=1) The pairwise sequence similarities were between 98.4-100% for the isolates belonging to *Firmicutes*, and 98.1-100% for the isolates belonging to *Proteobacteria* (Table I).

The colonies of moderately halophilic bacterial isolates on CMI agar plates were white, cream, yellow, orange and pink. While 41 of the isolates were Gram-positive, 36 were Gram-negative. Cell morphologies of the isolates were spherical, incomplete spiral, and rod-shaped (Table I). Our results were similar to other previous studies.[5,9]

All isolates grew in 5-12.5% salt concentrations; 18, 16, 10, 11, 6, 6, 4, 4 and 2 isolates were respectively grown at 3-20%, 3-25%, 3-12.5%, 3-15%, 0.5-20%, 0.5-15%, 0.5-25%, 5-25% and 0.5-12.5% salt concentrations. No growth was observed on salt-free media (Table I). Hence, these isolates were accepted as moderately halophilic bacteria.[5] Although all isolates were able to grow at between pH 6-8, none of them grew at pH 4 and pH 11. Twenty-one, nineteen, seventeen, fourteen, four and two isolates were respectively grown at pH 5-9, 6-8, 5-10, 6-10, 6-9 and 5-8. Twenty-five, thirty-two, ten, seven and three isolates were respectively grown at 20-40°C, 20-45°C, 4-40°C, 4-45°C and 28-45°C (Table I). Researchers stated that moderately halophilic bacteria may grow at 0.1-30% NaCl, 0-60°C and pH values of 5-10.[5]

All isolates were catalase positive. More than half of the isolates were oxidase positive. Production of indol, H_2S and N_2 were not common among the isolates. Biochemical test results revealed that all strains isolated from different skin samples were able to utilize a wide variety of organic compounds and carbon sources. Almost half of the isolates used citrate. Less than half of the isolates were Voges Proskauer positive. More than half of the isolates were methyl-red positive, reduced nitrate to nitrite, produced NH_3 (Table II). Positive catalase and oxidase reactions were also detected in *Thalassobacillus pellis* sp. nov., which is a new moderately halophilic bacterium isolated from salted hide. Similarly, indol formation and H_2S production by *Thalassobacillus pellis* sp. nov. was not observed. This isolate did not use citrate as a single carbon source and showed negative Voges Proskauer test. Reduction of nitrate to nitrite by this isolate was not observed.[26]

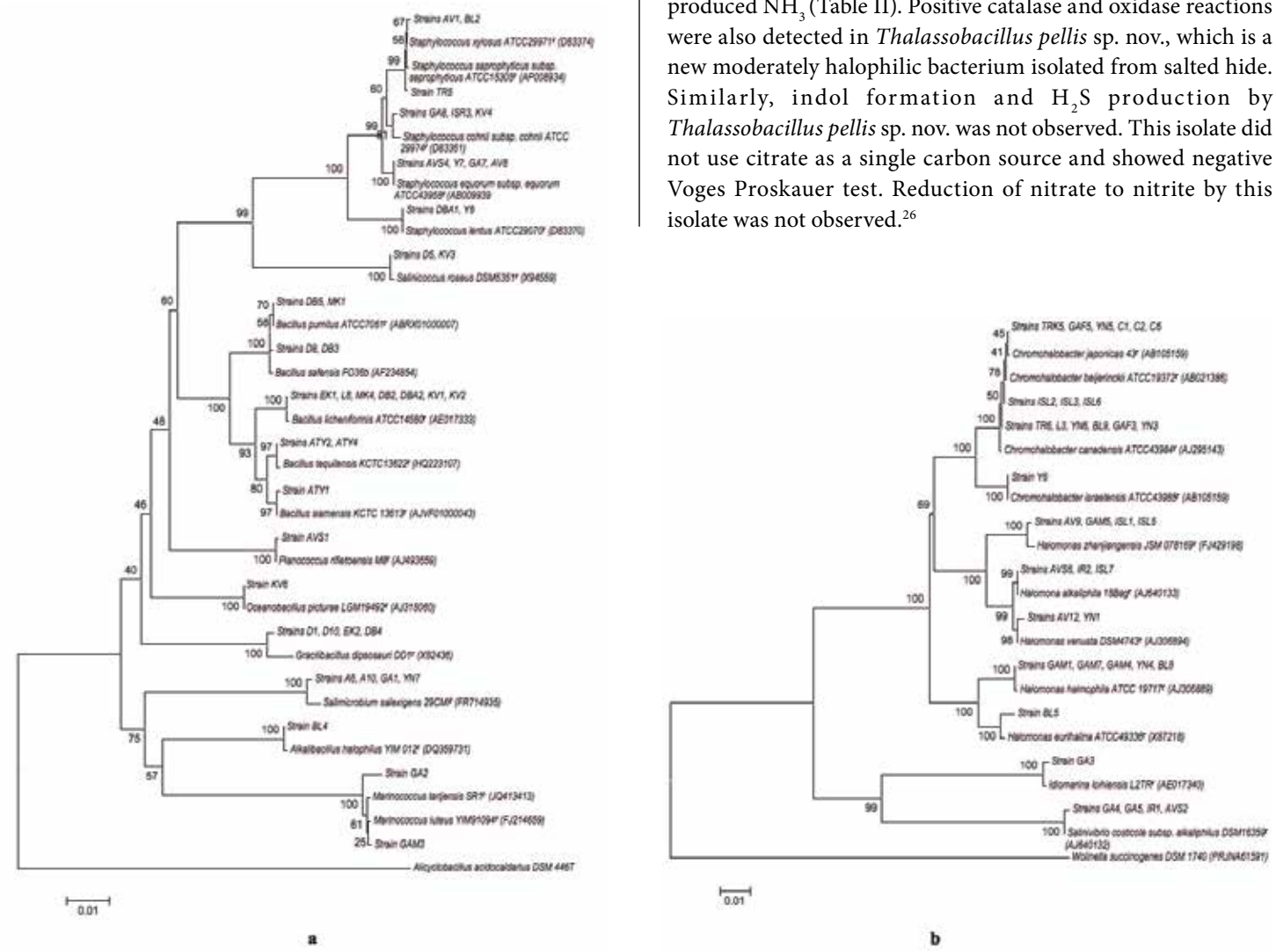

Figure 1. Phylogenetic trees, based on the 16S rRNA gene sequence comparison, showing the relationship of moderately halophilic bacterial isolates to the related species in the phyla *Firmicutes* (a) and *Proteobacteria* (b). The trees are based on the neighbor-joining method[20] and MEGA 4.0.[21]

Table I
Code, closest relative and similarity, morphological characteristic, NaCl, temperature and pH ranges for growth of the moderately halophilic bacteria studied

Code	Closest relative and similarity (%)	Pigmentation	Cell morphology	Motility	NaCl range (%)	Optimum NaCl (%)	Temperature range (°C)	Optimum temperature (°C)	pH range	Optimum pH
L8, EK1, MK4, DB2, DBA2, KV1, KV2	*Bacillus licheniformis* (98.4-99.8)	White	Rod	+	3-12.5	10	20-45	37	6-10	7
TR6, L3, YN6, BL9, GAF3, YN3	*Chromohalobacter canadensis* (99.6-99.9)	White	Rod	+	3-20	7.5-10	20-45	30-37	5-9	7
C1, C2, C6, YN5, GAF5, TRK5	*Chromohalobacter beijerinckii* (99.8-99.9)	Cream	Rod	+	3-25	7.5-10	4-40	30-37	5-9	7
DB5, MK1	*Bacillus pumilus* (98.7-99.8)	White	Rod	+	3-15	10	20-40	37	6-8	7
GAM1, GAM7, GAM4, YN4, BL8	*Halomonas halmophila* (98.1-99.8)	Yellow	Rod	-	3-25	10	20-45	37	5-10	7
AVS4, Y7, GA7, AV8	*Staphylococcus equorum* subsp. *equorum* (99.2-100)	Cream	Spherical	-	0.5-15	10	20-40	37	6-8	7
A6, A10, GA1, YN7	*Salimicrobium salexigens* (98.9-99)	Yellow	Spherical	-	3-25	7.5-10	20-40	37	5-10	7
D1, D10, EK2, DB4	*Gracilibacillus dipsosauri* (98.9-99.3)	White	Rod	+	0.5-20	10	20-45	37	5-9	7
GA4, GA5, IR1, AVS2	*Salinivibrio costicola* subsp. *alkaliphilus* (98.9-99.9)	Cream	Incomplete Spiral	+	3-20	10	4-45	37	5-10	7
AV9, GAM5, ISL1, ISL5	*Halomonas zhanjiangensis* (99.1-100)	Yellow	Rod	+	3-20	7.5-10	4-40	30	6-10	7
GA8, ISR3, KV4	*Staphylococcus cohnii* subsp. *cohnii* (99.1-100)	White	Spherical	-	3-15	10	20-45	37	6-8	7
AVS6, IR2, ISL7	*Halomonas alkaliphila* (99.6-99.9)	White	Rod	-	3-20	10	20-45	37	5-10	8
ISL2, ISL3, ISL6	*Chromohalobacter japonicus* (99.6-99.8)	Cream	Rod	+	5-25	10	20-40	37	6-9	7-8
AV1, BL2	*Staphylococcus xylosus* (99.4-99.6)	Yellow	Spherical	-	3-12.5	10	20-40	37	6-8	7
Y8, DBA1	*Staphylococcus lentus* (100)	Yellow	Spherical	-	3-15	10	20-40	37	6-8	7
ATY2, ATY4	*Bacillus tequilensis* (99.9)	Yellow	Rod	+	0.5-15	10	28-45	37	6-8	7

Table I continues on following page.

Table I continued.

D8, DB3	*Bacillus safensis* (99.7-99.8)	Cream	Rod	+	0.5-12.5	10	20-45	37	5-8	7
KV3, D5	*Salinicoccus roseus* (99.4-99.6)	Pink	Spherical	-	0.5-25	10	20-40	37	5-9	7
YN1, AV12	*Halomonas venusta* (98.5-99.5)	Cream	Rod	-	3-15	10	20-40	37	6-10	7
TR5	*Staphylococcus saprophyticus* subsp. *saprophyticus* (99.8)	Yellow	Spherical	+	3-15	10	20-40	37	6-8	7
ATY1	*Bacillus siamensis* (99.8)	White	Rod	-	3-12.5	10	4-45	37	5-9	7
AVS1	*Planococcus rifietoensis* (99.8)	Orange	Rod	+	3-15	10	20-40	37	6-10	8
BL4	*Alkalibacillus halophilus* (99.8)	White	Rod	+	5-25	10	28-45	37	6-9	7-8
GA2	*Marinococcus luteus* (99.2)	Yellow	Spherical	+	0.5-25	10	20-40	28-30	6-8	7
GAM3	*Marinococcus tarijensis* (99.6)	Yellow	Spherical	+	3-25	10	20-45	37	6-8	7
KV6	Oceanobacillus picturae (100)	Cream	Rod	+	0.5-20	10	20-40	30	6-8	7
BL5	*Halomonas eurihalina* (99.2)	Cream	Rod	+	0.5-25	10	4-45	37	5-9	7
Y6	*Chromohalobacter israelensis* (99.9)	Cream	Rod	+	3-20	7.5-10	20-45	30-37	5-9	7
GA3	*Idiomarina loihiensis* (99.6)	Cream	Rod	+	0.5-20	7.5-10	4-45	37	5-10	7

More than half of the isolates produced acid from sucrose, D-galactose, D-trehalose, D-melibiose, D-xylose, L-arabinose, D-cellobiose, fructose, lactose and maltose. Fairly high percentage of the isolates produced acid from D-glucose (95%) and D-mannose (90%) (Table II). *Thalassobacillus pellis* sp. nov. produced acid cellobiose, D-galactose, D-glucose, lactose, maltose, D-mannose, sucrose, trehalose and D-xylose.[26] Another new moderately halophilic bacterium, *Salimicrobium salexigens* sp. nov., produced acid from D-glucose, maltose, sucrose, D-galactose and D-trehalose.[27] It was stated that moderately halophilic bacteria grown in environments containing plants and animals may use many different nutrients.[28] Our experimental results supported this information, and our isolates used a wide variety of carbon sources (Table II).

While 60%, 43%, 39%, 26%, 23%, 17%, and 10% of the isolates respectively produced protease, lipase, β-galactosidase, amylase, caseinase, DNase, and lecithinase, 12% of isolates produced urease and cellulase. None of the isolates produced pullulanase, xylanase and phospholipase enzymes (Table II). Detection of 61 isolates producing industrially important enzymes in this study may be related to different countries' salt samples used in the skin preservation (Table II).

Table II

Biochemical and enzymatic results of moderately halophilic isolates.

Characteristics	*B. licheniformis*	*C. canadensis*	*C. beijerinckii*	*B. pumilus*	*H. halmophila*	*S. equorum* **subsp.** *equorum*	*S. salexigens*	*G. dipsosauri*	*S. costicola* **subsp.** *alkaliphilus*	*H. zhanjiangensis*	*S. cohnii* **subsp.** *cohnii*	*H. alkaliphila*	*C. japonicus*	*S. xylosus*	*S. lentus*	*B. tequilensis*	*B. safensis*	*S. roseus*	*H. venusta*	*S. saprophyticus* **subsp.** *saprophyticus*	*B. siamensis*	*P. rifietoensis*	*A. halophilus*	*M. luteus*	*M. tarijensis*	*O. picturae*	*H. eurihalina*	*C. israelensis*	*I. loihiensis*	**Positive isolate numbers**	**Percentage of positive isolates**
Isolate numbers	7	6	6	2	5	4	4	4	4	4	3	3	3	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1		
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	77	100
Oxidase	+	-	+	+	+	-	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	-	-	+	+	-	+	53	69
Indole production	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	9	12
Citrate utilization	+	-	+	+	+	-	-	-	-	+	-	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	36	47
Methyl-red test	+	+	+	-	+	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	-	-	+	+	-	+	50	65
Voges-Proskauer test	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	18	23
Production of H_2S	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	6	8
Nitrate reduction	+	+	+	-	+	+	+	+	-	+	-	+	+	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	+	60	78
Production of N_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	5	6
Production of NH_3	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	67	87
Production of acid from carbon sources																															
Sucrose	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	+	-	-	+	+	-	55	71
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	72	95
D-galactose	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+	-	-	+	+	-	-	+	+	+	+	+	-	60	78
D-trehalose	+	-	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	59	77
D-melibiose	+	+	+	-	+	+	-	+	+	-	-	+	+	-	+	+	+	-	-	-	+	-	-	+	+	+	-	-	-	52	68

Table II continues on following page.

Table II continued.

D-mannose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+	69	90
D-xylose	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-	+	+	-	+	-	+	+	-	+	-	+	-	+	+	61	79
L-arabinose	+	+	+	+	+	+	-	+	-	+	+	-	+	+	-	+	+	-	-	-	+	+	-	+	+	+	+	+	-	57	74
D-cellobiose	+	+	-	+	+	+	-	-	+	-	+	+	+	-	-	+	+	-	-	-	+	-	-	+	+	-	+	-	-	45	58
Fructose	+	-	-	+	+	-	+	+	-	+	+	+	-	+	-	+	-	-	-	+	+	-	-	-	-	+	+	+	+	42	55
Lactose	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	-	-	+	+	+	-	59	77
Maltose	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	59	77
Enzymatic activities																															
Proteaz	+	+	-	+	-	+	-	+	+	-	-	-	+	+	-	+	+	+	-	+	+	+	-	+	+	-	+	+	+	46	60
Lipase	+	+	-	+	-	-	-	+	+	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	+	+	+	33	43
β-galactosidase	+	-	-	+	-	+	-	+	-	-	+	-	-	+	-	+	+	-	-	+	-	+	-	+	-	+	-	-	-	30	39
Amylase	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	20	26
Caseinase	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	18	23
DNase	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	13	17
Urease	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	9	12
Cellulase	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	9	12
Lecithinase	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	8	10
Pullulanase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
Xylanase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
Phospholipase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0

Enzymatic activities of moderately halophilic bacteria were also reported in different studies.[8-10] Researchers isolated 9848 colonies of moderately halophilic bacteria from saline environments in Spain. Researchers stated that 269, 118, 207, 201 and 97 of isolates were respectively amylase, DNase, lipase, protease, and pullulanase producers. However, none of the isolates produced xylanase.[8] These researchers also stated that environmental isolates belong to the genera *Salinivibrio* and *Halomonas* (Gram-negative), *Bacillus* and *Salibacillus* (Gram-positive) produced most hydrolytic enzymes.

Investigators isolated 231 moderately halophilic bacteria from Howz Soltan playa in Iran. In that study, lipases, amylases, proteases, inulinases, xylanases, cellulases, pullulanases, DNases, and pectinases were produced by 195, 177, 100, 95, 92, 68, 65, 33, and 28 moderately halophilic bacterial isolates, respectively.[9]

In another study, researchers isolated 108 moderately halophilic bacteria from soil, sea water and water samples collected from saline environments of India. Investigators mentioned that 33, 38 and 54 moderately halophilic isolates produced amylase, lipase and protease, respectively.[10] After identification of 21 isolates producing enzymes, *Geomicrobium halophilum*, *Staphylococcus xylosus*, *Virgibacillus halodenitrificans*, *Bacillus lehensis* and *Oceanobacillus iheyensis* were detected as protease

producers. While *Halobacillus kuroshimensis*, *Halobacillus trueperi* and *Halobacillus kuroshimensis* produced amylase, lipase was produced by *Marinobacter litoralis*, *Chromohalobacter israelensis*, *Geomicrobium halophilum* and *Halomonas salina*.[10]

The enzymes that hydrolyze gelatin, casein, DNA and pullulan have been also detected in *Thalassobacillus pellis* sp. nov. isolated from salted hide.[26] DNase production by salted hide isolate *Salimicrobium salexigens* sp. nov. was also stated.[27]

In the present study, isolates exhibiting most combined activities were belong to the genera *Bacillus*, *Staphylococcus, Gracilibacillus, Salinicoccus, Salinivibrio* and *Idiomarina*. Although a variety of genera members produced protease, lipase, β-galactosidase, amylase and DNase, a few genera members produced urease, lecithinase, caseinase and cellulase in our experiment. Especially *B. licheniformis* and *B. tequilensis* showed six different combined activities (Table II). As known, bacterial proteases, amylases and lipases are used in the largest quantity in especially leather and detergent industries. *B. licheniformis* was recognized as the best producer of these enzymes.[1] Our results also proved that moderately halophilic *B. licheniformis* was the best enzyme producer. Besides this organism, *B. tequilensis* might be a good candidate for production of industrial enzymes.

Conclusion

In this study, we especially investigated the industrial importance of moderately halophilic bacteria found on salted sheep skins and characterized these isolates using conventional biochemical tests and molecular methods. Our experimental results showed that moderately halophilic bacterial isolates were closely similar to species of the genera *Staphylococcus*, *Salimicrobium*, *Bacillus*, *Salinicoccus*, *Planococcus*, *Alkalibacillus*, *Gracilibacillus*, *Oceanobacillus*, *Marinococcus*, *Halomonas*, *Salinivibrio*, *Chromohalobacter* and *Idiomarina*. Combined hydrolytic activities were detected at *Bacillus licheniformis, B. pumilus, B. tequilensis, B.safensis, B.siamensis, Chromohalobacter canadensis, C.israelensis, Staphylococcus equorum* subsp. *equorum, S. saprophyticus* subsp. *saprophyticus, S.cohnii* subsp. *cohnii, S. xylosus, Gracilibacillus dipsosauri, Salinivibrio costicola* subsp. *alkaliphilus, Salinicoccus roseus, Planococcus rifietoensis, Marinococcus luteus, M.tarijensis, Oceanobacillus picturae, Halomonas eurihalina* and *Idiomarina loihiensis*. Protease, lipase, β-galactosidase, amylase, caseinase, DNase, urease, cellulase and lecithinase enzymes produced by these isolates may be used in baking, paper, starch, textile, laundry, dry cleaning, meat, medicine, candy, soft drink, milk, chemical and leather. More detailed research is currently under way to characterize these enzymes and determine their biochemical properties.

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