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Innovative Green Chemical Production by Novel Egyptian Aquatic Bacterial Isolates

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Over the last decade, lactic acid has attracted great attention as a natural preservative in food and cosmetics. Poly–lactic acid can be used in bioplastic production that can serve as a biobased alternative to oil–derived plastics such as polyethylene and polystyrene. In this study, a total of 16 novel marine lactic acid bacteria isolates were selected from samples collected along the Alexandrian Mediterranean Sea coast of Egypt. Both biochemical characteristics of LAB isolates supported by 16S rRNA gene sequence analysis and NCBI online database tool showed that the obtained isolates belonged to the genera *Pediococcus* and *Lactobacillus*. The results of lactic acid fermentation using those marine isolates revealed improved homo–lactic acid fermentation (100% optically pure lactic acid produced) from low xylose concentration after 24 h at initial pH 5.0 and 50°C. To the best of our knowledge, this is the first report on marine bacteria as candidates for the production of green chemicals in the Mediterranean Sea in Egypt.

Key words: Marine lactic acid bacteria, Mediterranean Sea, Optically pure lactic acid, *Pediococcus acidilactici*

INTRODUCTION

Lactic acid, first discovered by the Swedish chemist Scheele in 1780, is present in many foods, either naturally or as a product of *in situ* microbial fermentation such as Sauerkraut, yoghurt, butter milk, sourdough breads, and many other fermented foods (Reddy *et al*., 2008). Lactic acid is also produced in muscles of mammals during glycolysis and is involved in the Kreb's cycle through pyruvic acid and acetyl–CoA (Fambri *et al*., 1997). US FDA (Food and Drug Administration) classified lactic acid as GRAS (Generally Recognized As safe) for use as a food additive and has a long history of uses in food, chemical, cosmetic, and pharmaceutical industries (Wee *et al*., 2006).

There is an elevated need for lactic acid as a green chemical for the production of biopolymer poly–lactic acid (PLA) synthesized from isomers either L(+)– or D(–)–lactic acid Abdel–Rahman *et al*. (2011a, b). The optical purity of lactic acid is crucial to the physical properties of a high crystalline PLA for packaging applications and offers a good shelf life, rather than racemic DL–lactic acid (Södergård and Stolt, 2002; Auras *et al*., 2004). Fossil fuel–derived plastics are non–renewable, often threaten public health, have devastating impacts on marine life, and increase dependance on imported fossil fuel–based feedstocks. The development of bioplastics holds great promise to resolve majority of these sustainability problems, offering the possibility of renewability, biodegradation, and a path away from harmful additives. Therefore, the biotechnological production of lactic acid has recently received a significant amount of interest, since it offers an eco–friendly alternative to environmental pollution caused by petrochemical industry and used as the next generation of plastic materials (Hamdan and Sonomoto, 2011).

Currently, the worldwide consumption of lactic acid is estimated to be 130 000–150 000 (metric) ton per year, and the commercial prices of food grade lactic acid range between 1.38 US$/kg for 50% purity and 1.54 US$/kg for 88% purity (Bozell and Petersen, 2010). Technical grade lactic acid with 88% purity has been evaluated to be about 1.59 US$/kg (Wee *et al*., 2006), while lactic acid consumption in chemical applications, including PLA polymer and new green solvents, such as ethyl lactate, is expected to increase 19% per year (Jarvis, 2003).

Furthermore, chemical production of lactic acid from petrochemical resources always results in racemic mixture of DL–lactic acid, which is a major disadvantage of this approach (Sobrun *et al*., 2012). Conversely, lactic acid fermentation using lactic acid bacteria (LAB) shows superiority in terms of the utilization of renewable biomass, low production temperature, low energy consumption, and yielding optically pure lactic acid by choosing an appropriate strain (Tashiro *et al*., 2011).

LAB are a group of Gram–positive, anaerobic, cata-

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lase–negative and non–sporing bacteria (Orla–Jensen, 1919). LAB exhibit enormous capacity to degrade different carbohydrate substrates from surplus agricultural crops and industrial wastes in the production of various value added products (Vishnu *et al*., 2006). Exploration of aquatic microbial diversity is clearly a topic of considerable importance and interest because there are few reports on isolation of lactic acid–producers from marine resources (Ringø and Gatesoupe, 1998; Švec *et al*., 2001 and 2005; Sistek *et al*., 2012). A few strains of LAB were previously isolated from marine environment, mostly from the Pacific Ocean region of Japan (Franzmann *et al*., 1991). For example, the genus *Marinilactobacillus* has been isolated from the coastal sub–seafloor sediments of the Okhotsk sea in Japan (Inagaki *et al*., 2003). Also, many *Carnobacterium*–like strains have been retrieved from the Nankai Trough, in south–east of Japan (Newberry *et al*., 2004). A novel species, *Marinilactobacillus piezotolerans* has been obtained from sediment core collected at 4.15 m below seafloor from water depth 4790.7 m at the Nankai Trough (Toffin *et al*., 2005). On the other hand, *Carnobacterium funditum* and *C. alterfunditum* are reported to be isolated from the Ace lake in Antarctica (Spielmeyer *et al*., 1993), while *Pediococcus acidilactici, Weissella paramesenteroides, Ped. pentosaceus* and *Enterococcus faecium* where isolated from sediments, water and seaweeds from the west coast of India (Shobharani *et al*., 2012). All previous reports indicated that Gram–positive bacteria including mainly the *Firmicutes* and *Bacillus* are the most frequent genus, followed by Actinobacteria and *Gammaproteobacteria* which were clearly dominant among isolates from sediments of the Mediterranian Sea (D'hondt *et al*., 2004; Stevens *et al*., 2007). Hence, analysis of microbial diversity from aquatic environment may help in isolating and identifying potential lactic acid producers (Das *et al*., 2006). In the present study, several new thermotolerant LAB strains were isolated from the Egyptian Mediterranean Coast of Alexandria, and have efficiently produced optically pure L(+)–lactic acid. This study could be considered the first report of utilizing microbial isolates from the Egyptian marine habitats for green chemical production.

MATERIALS AND METHODS

Screening for novel Egyptian marine LAB isolates

Twenty–seven marine samples; sediments, algae, sponge and corals, were collected from the sea coastal line of Alexandria City, Egypt, and cultivated on de Man, Rogosa and Sharpe (MRS) (Oxoid, UK) broth medium prepared in filtered seawater under anaerobic conditions and incubated at 37°C for 3–5 days. Selected Gram–positive pure isolates with catalase–negative activity were maintained in MRS broth for regular use or stocked in 30% glycerol and stored at –20°C until used (Hamdan *et al*., 2016). Furthermore, cell morphology was observed by using Fluorescence Microscopy at 1,000× magnification (Olympus BX 41, Tokyo, Japan).

Characterization and identification of marine LAB

Physiological characteristics of LAB isolates were tentatively determined by using API 50 CHL test kits (bioMérieux, Marcy l'Étoile, France) as described by the manufacturer and scored after 24 and 48 h of incubation at 37ºC. Interpretation of the results was facilitated using the computer–aided database APIWEB™ V.5.0. software (Khalil *et al*., 2007).

Furthermore, isolates were identified by 16S rDNA and sequenced following the method described by Abdel–Rahman *et al*. (2011a). Partial 16S rDNA region of the isolate, corresponding to positions 8–1510 of *Escherichia coli* 16S rDNA, was analyzed using a set of universal primers, 8UA and 1510r. The sequences of the primers are as follows: 8UA, 5'–AGAGTTTGATCCTGGCTCAG–3'; and 1510r, 5'–GGTTACCTTGTTACGACTT–3'. Total genomic DNA was extracted from cells treated with lysozyme (Seikagaku, Tokyo, Japan) using the Mag Extractor Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Genomic DNA was used as a polymerase chain reaction (PCR) template. PCR was performed using Taq DNA polymerase (Promega, Madison, WI, USA) under the following conditions: denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 90 s. The amplified products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Finally, DNA sequencing analysis was performed by FASMAC Co., Ltd (Kanagawa, Japan). Similarity search was performed in the GenBank database using the online tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Analysis of microbial diversity using PCR–DGGE method

The community structure of total LAB associated with sediment samples (as well as in the bulk sea water) was assessed by polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE), following the procedures described by Hardoim *et al*. (2009). Genomic DNA of each marine sample was extracted in duplicate DNA isolation kit (MO BIO Laboratories, Inc., USA) according to the manufacturer's manual and pooled together for subsequent molecular analysis and stored at –20°C.

PCR was performed using primer pairs, WLAB1 and WLAB2, described by López *et al*. (2003). After an initial denaturation at 94°C for 5 min, 35 cycles of 1 min at 95°C, 1 min at 53°C, and 2 min at 72°C were performed. A final extension step of 10 min at 72°C was used to finish the reaction. PCR–amplified products were purified with a Gel Advanced™ Gel Extraction System according to the manufacturer's instructions and checked by electrophoresis in 1% agarose gels after staining with ethidium bromide and visualized with UV transillumination. Blocks of polyacrylamide gels which contained the selected DGGE bands were excised and subsequently incubated overnight in 20 µl of sterile and pure water at 4°C to allow DNA bands diffuse to the liquid. One µl of this elution was re–amplified by PCR and subjected to DNA sequencing (González–Arenzana *et al*., 2013).

Sequences were aligned in GenBank using the online tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the closest known relatives of the partial 16S rDNA sequences obtained. Phylogenetic tree was constructed using MEGA V.6 with the neighbor–joining method with 1,000 bootstrapping to estimate the confidence of the tree topologies (Wang *et al*., 2014).

Scanning electron microscopy (SEM)

Selected LAB isolates were visualized by scanning electron microscopy (SEM), following the procedure described by Kokkinos *et al*. (1998). Briefly, LAB isolates were cultured in MRS broth, centrifuged at 6,000 × g for 10 min and washed 3 times with 0.85% NaCl solution. Cell pellets were fixed by 0.25% glutaraldehyde (in Na–phosphate, pH 7.2), incubated at room temperature for 30 min and washed 3 times with Na–phosphate buffer. Pellets were collected by centrifugation, dehydrated for 10 min by different ethanol volumes starting; 30%, 50%, 70%, 80%, 90% and 100% and incubated in 100% ethanol for 1 h. After drying, SEM stubs were coated with gold and examined with JEM–100XJoel electron microscope in the Faculty of Science, *Alexandria* University, Egypt.

Lactic acid production

To investigate lactic acid production under optimum culture conditions, isolates of marine LAB were subjected to cultivation on MRS broth medium containing different carbon sources (D–glucose, or D–xylose), different initial pH values (3.5~7.0) and wide temperature range (5~50°C) under anaerobic conditions for 72 h. Fermentations were performed with agitation at 200 rpm, in a 1–L jar fermenter with a 400–ml working volume (Abdel–Rahman *et al*., 2011b). Samples were periodically withdrawn after 24, 48 and 72 h and lactic acid yield (g lactic acid/ g consumed sugar) was calculated. L–lactic acid optical purity (%) was measured using the following formula:

$$\text{L–lactic acid optical purity (\%)} = \frac{(\text{L–LA concentration} - \text{D–LA concentration})}{(\text{L–LA concentration} + \text{D–LA concentration})} \times 100$$

LA, lactic acid.

Analytical methods

Xylose, glucose and any fermentation products (lactic acid, acetate and/or ethanol) were analyzed using high–performance liquid chromatography (HPLC) system (US HPLC–1210, Jasco, Tokyo, Japan) equipped with a SUGAR SH–1011 column (Shodex, Tokyo, Japan). Aliquots from each sample were centrifuged at 6,000 × g for 10 min at 4°C. Thereafter, the supernatant was filtered using a membrane filter (Dismic–13HP, 0.45 μm, Advantec, Tokyo, Japan) and finally injected in the HPLC system under the following conditions: column temperature of 50°C, 3 mM $HClO_4$ as the mobile phase at a flow rate of 1.0 ml/min and an injection volume of 20 μl. The concentrations of residual sugars and fermentation products were calculated using calibration curves obtained by using standard solutions. The optical purity of lactic acid was measured using a BF–5 biosensor (Oji Scientific Instruments, Hyogo, Japan) according to the manufacturer's protocol.

RESULTS

A total of 16 LAB isolates were obtained from 27 marine samples collected from the Alexandria sea coast, Egypt. The selected isolates showed no haemolytic activity on sheep blood and horse blood agar plates (Eiken Chemical, Tokyo, Japan), indicating the safety of those strains. Based on both physiological characteristics and 16S rDNA gene sequence analysis of each isolate, 14 isolates showed 99% similarity to the genus *Pediococcus* and were primarily identified as *P. pentosaceus* (10 isolates), *P. lolii* (3 isolates) and *P. acidilactici* (1 isolate). Furthermore, two isolates showed 99% identity to *Lactobacillus plantarum*. In addition, microscopic examination of those isolates confirmed their Gram–positive reaction with non–spore formation and exhibited cocci and rod–shaped morphology by SEM micrographs

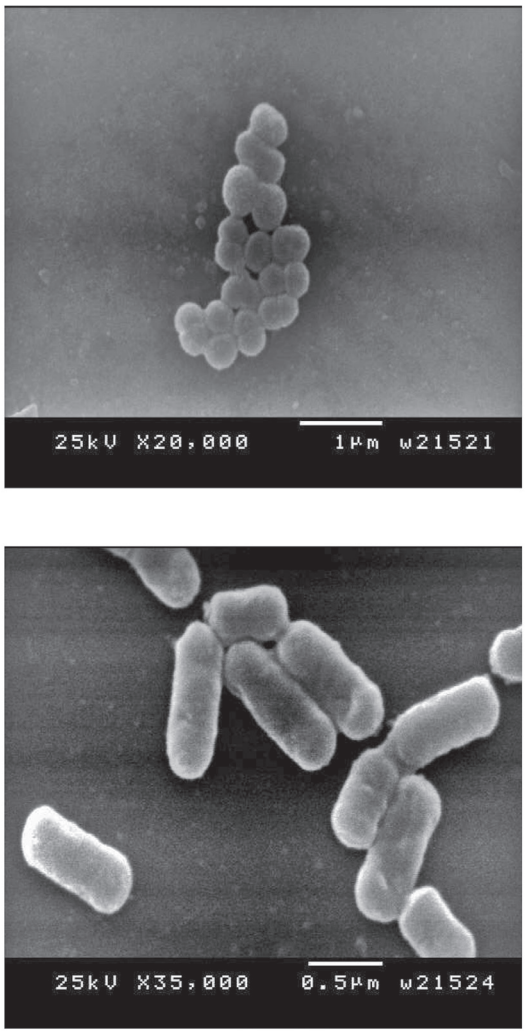

Fig. 1. SEM micrographs of two marine LAB isolates (A) *Pediococcus* and (B) *Lactobacillus*.

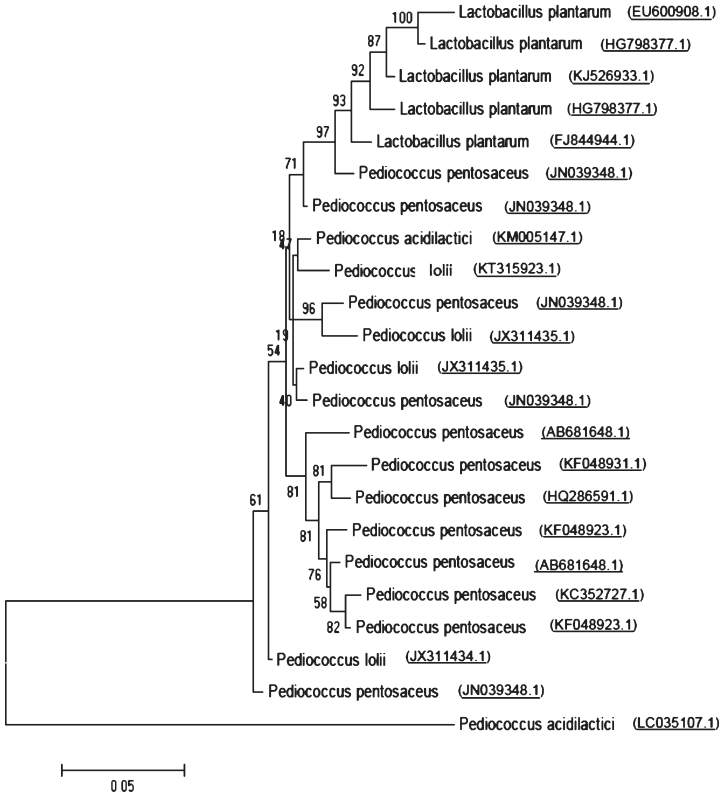

Fig. 2. The phylogenetic tree showing the relative position of the LAB isolates. It was constructed using MEGA V.6 and shows the relative position of the LAB isolates retrieved from sediment samples based on their 16S rDNA partial sequences, using the neighbor-joining method with 1,000 bootstrapping. The scale bar corresponds to 0.05 units of the number of base substitutions per site, each terminal node is labeled with the respective species name and the numeric GenBank identifier number.

(Fig. 1).

By applying PCR–DGGE technique, furthermore, phylogenetic tree of total LAB maintained from marine samples was constructed using MEGA V.6 with the neighbor–joining method (Fig. 2). The results revealed that the molecular fingerprinting of both cultivable plus non–cultivable LAB populations provided evidence of the dominance of the genera *Pediococcus* and *Lactobacillus* in all marine samples.

The time–course batch fermentations conducted over 72 h incubation periods under different culture conditions showed that maximum L–lactic acid production (93% optical purity) was obtained at 50°C in 10 g/l xylose with absence of any other by–products (only one HPLC peak of fermentation product appeared) (Fig. 3). However, at temperature range 5~30°C, the results revealed that marine LAB isolates did not show either good cell proliferation or high lactic acid production. Furthermore, at pH value of 5.0, xylose consumption (Ⅱ2, Ⅱ4, Ⅱ5, Ⅲ3) was slightly higher and led to improved L–lactic acid fermentation (100% optical purity) after 24 h, than pH 4, and did not deplete until the end of fermentation (72 h). However, at pH 5.0, although all glucose was utilised (Ⅲ2, Ⅲ5) after 24 h, lactic acid yield did not exhibit maximum values, 0.89 and 0.88, respectively (Table 1). Conversely, lactic acid yield declined by increasing the pH till 7.0, with complete sugar consumption (Data not shown).

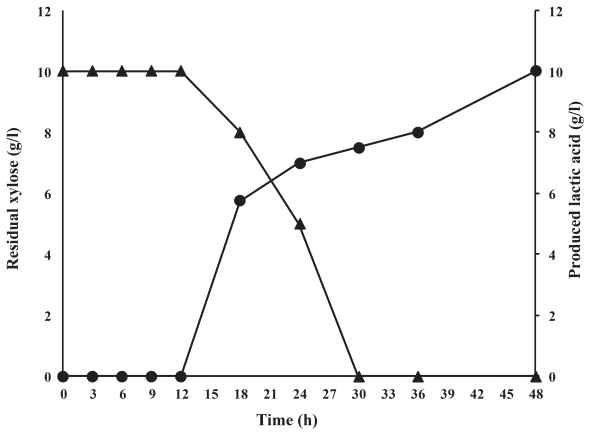

Fig. 3. Time course of lactic acid fermentation by the marine LAB isolate (Ⅱ3) using 10 g/l xylose at 50°C. Symbols; produced lactic acid (closed circle), residual xylose (closed triangle).

Our results indicate that capabilities of the novel marine Egyptian LAB to utilize xylose. In contrast to other reported *Pediococuus* or *Lactobacillus* strains, the novel marine isolates could efficiently ferment xylose to L–lactic acid homofermentatively at 50°C with high yields of approximately 1.0 g/g with no other by–products.

Table 1. The effect of different pH values and carbon sources on L-lactic acid batch fermentations

Isolate Designation	pH 4			pH 5		
	Lactic acid (g/l)	Consumed sugar (g/l)	YLA/TS (g/g)	Lactic acid (g/l)	Consumed sugar (g/l)	YLA/TS (g/g)
I3 X	8.48	9.9	0.85	8.41	8.7	0.96
I4 X	8.28	9.58	0.86	8.57	9.54	0.89
I5 X	11.72	13.79	0.84	10.04	9.37	1.07
II2 X	7.31	8.27	0.88	8.33	9.78	0.85
II3 X	8.74	9.95	0.87	10	9.76	1.02
II4 X	7.49	8.54	0.87	8.55	9.37	0.91
II5 X	7.43	8.8	0.84	8.85	9.65	0.91
III1 G	15.06	14.67	1.02	19.89	18.7	1.06
III2 G	15.45	15.04	1.02	17.8	20	0.89
III3 X	8.55	8.92	0.95	10.93	10	1.09
III3 G	16.24	14.93	1.08	17.79	16.51	1.07
III5 G	17.34	18.29	0.94	17.7	20	0.88

X and G represent xylose and glucose, respectively. YLA/TS represents the yield of lactic acid to total consumed sugar. Cultivation was carried out at 50°C for 24 h. The experiment was carried out in triplicates and the average data are shown.

DISCUSSION

Due to concerns about the availability of recoverable fossil fuel reserves and the environmental problems caused by the use of those fuels, research intensiveness on the production of green chemicals has grown several folds over the past decade (Bauer *et al*., 2016). Lactic acid production has attracted a great deal of interest due to its potential use as a raw material in the production of the biodegradable polymer PLA. Of the 150 000 tons of the aunral production of lactic acid produced worldwide every year about 90% are made by bacterial fermentation and the rest is produced synthetically by the hydrolysis of lactonitrile (Shibata *et al*., 2007).

In the meantime, the isolation and screening of microorganisms from natural sources represents the most potent means of obtaining beneficial and genetically stable strains for industrially important products (Adnan and Tan, 2007).

Generally, the optimum temperatures for lactic acid production by LAB are ≤40°C; nevertheless, 43°C was the optimum temperature described in cases of *Enterococcus mundtii* QU 25 (Abdel–Rahman *et al*., 2011b) and *Lactobacillus delbrueckii* subsp. *lactis* QU 41 (Tashiro *et al*., 2011). Using thermophilic bacteria has many economic advantages such as saving energy and reduced contamination chances for both saccharifying enzymes and lactic acid producing bacteria (Abdel–Rahman *et al*., 2015).

Although many studies have been describing homo–lactic acid production using xylose by the non–LAB, thermophilic *Bacillus* strains (Wang *et al*., 2010; Ye *et al*., 2013), to the best of our knowledge, it should be emphasized that this is the first report of efficient LAB–mediated lactic acid production from Egyptian marine resources under thermophilic conditions (≥50°C).

Since to date, only few superior LAB were isolated from the seas and oceans for lactic acid production, as an important green chemical. Novel Egyptian marine LAB isolates proved to be a promising strain enabling production of L–lactic acid from lignocellulose–derived sugars (glucose, xylose, arabinose, mannose, galactose, and cellobiose). From an economical point of view, screening for economical alternative raw materials and nutrients aiming to reduce production cost of added–value products is of superior importance. This study shows the potential of Egyptian marine isolates in reaching the demand for lactic acid, meeting its widest applications nowadays.

AUTHORS CONTRIBUTIONS

Waill A. Elkhateeb and Amira M. Hamdan carried out the experiments. Kenji Sonomoto, Takeshi Zendo, and Yukihiro Tashiro, conceived the study, and Kenji Sonomoto was in charge of overall direction and planning. Waill A. Elkhateeb and Amira M. Hamdan wrote the manuscript in consultation with Kenji Sonomoto and Takeshi Zendo. All authors discussed the results and commented on the manuscript.

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CONFLICT OF INTEREST

No conflict of interest is declared.

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