Carbohydrate Polymers 92 (2013) 1172–1176

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

A novel method for promoting antioxidant exopolysaccharidess production of *Bacillus licheniformis*

Yaowei Fang, Shu Liu, Mingsheng Lu, Yuliang Jiao, Shujun Wang*

School of Marine Science and Technology, Huaihai Institute of Technology, Lianyungang 222005, China

ARTICLE INFO

Article history:
Received 5 August 2012
Received in revised form 3 October 2012
Accepted 5 October 2012
Available online 24 October 2012

Keywords:
Marine bacterial
Bacillus licheniformis
Antioxidant extracellular polysaccharides
Promotion
Organic solvent stress

ABSTRACT

A novel method was described for improving the production of antioxidant extracellular polysaccharides from *Bacillus licheniformis*. Firstly, the tolerances of the strains to the organic solvents were investigated. Wild type strain of *B. licheniformis* OSTK95 and mutant strain UD061 can grow in a liquid medium in the presence of organic solvents with the log *P* value equal to or higher than 3.5 and 3.1, respectively. Secondly, the effects of different concentrations of n-hexane and xylene treatment on the extracellular polysaccharides excretion of both strains were studied. The maximum yield of the extracellular polysaccharides of *B. licheniformis* OSTK95 was 68.59 mg L^{-1} after treated by 10% n-hexane or 1% xylene for 3 h, while the maximum yield of the extracellular polysaccharides of strain UD061 was 185.01 mg L^{-1} after treated by 12.5% n-hexane or 5% xylene for 3 h. Finally, the continuous passage experiment showed that the strains have high genetic stability.

1. Introduction

Microbial exopolysaccharides (EPS) are biosynthetic polymers mainly consisting of carbohydrates secreted by microorganisms (Harrah, Panilaitis, & Kaplan, 2007; Ravella et al., 2010). Microbial EPSs have been utilized as ingredients in food products, pharmacy, petroleum industry and emulsification of crude oil, hydrocarbons, vegetable, mineral oils and bioremediation agents in environment management system (Kuzma et al., 2012; Wang, Huang, Liang, Fang, & Wang, 2011).

Although in recent years, various polysaccharides of microbial derivation have been reported, there is a demand for novel EPSs that impart improved physical or chemical characteristics (Luo et al., 2009). Marine microorganisms have developed unique metabolic and physiological capabilities to thrive in extreme habitats and produce novel metabolites which are not often present in microbes of terrestrial origin (Fang et al., 2010; Satpute, Banat, Dhakephalkar, Banpurkar, & Chopade, 2010). Therefore, the marine microorganisms provide a magnificent opportunity to discover newer compounds include EPSs (Fang et al., 2010). Moreover, studies of bacteria growing in marine sediments, aggregates, and detrital particles have shown that nearly all the cells are surrounded by extracellular polymeric material, and many of these cells are enclosed EPSs. Various novel EPSs producing microorganisms have been isolated and characterized from marine samples (Hassler, Alasonati, Mancuso Nichols, & Slaveykova, 2011; Satpute et al., 2010). Unfortunately, only few of those microorganisms have been commercially exploited because of the low yields of EPSs production by the majority of microorganisms (Lima et al., 2008; Lin, 2010; Liu et al., 2010; Luo et al., 2009). Although cultivation conditions optimization or mutation breeding were utilized to improve the yield of EPSs from microorganisms, the yields of polysaccharide production of some microorganisms are still low (Fang et al., 2010; Liu et al., 2010). In order to better exploit and utilize this kind of resource, it is essential to further investigate the technology to improve the productivity of EPSs from microorganisms.

Bacillus licheniformis OSTK95 is a marine bacterium isolated from marine mud samples collected from a crude water contaminated environment located in Lianyungang, Jiangsu Province, China. Recently, we reported the culture conditions and antioxidant activity in vitro of EPS from the strain (Fang et al., 2010). The crude EPSs exhibited strong scavenging activities on superoxide and hydroxyl radicals in vitro. In our previous report, the effects of various composition of fermentation medium on EPSs production were investigated by single factor method and optimized by orthogonal array experiments. A higher EPSs production mutant, named UD061, was obtained from initial *B. licheniformis* OSTK95 by means of UV and DES mutagenization. Polysaccharides are believed to protect bacterial cells from organic compounds stresses, and to produce biofilms, thus to enhance the chances of the cells to colonize special ecological niches. However, reports about the research about the effects of the organic solvent on yields of the EPSs from microorganisms seem to be scarce.

* Corresponding author. Tel.: +86 0518 85895430; fax: +86 0518 85806171.
E-mail address: abioedu@gmail.com (S. Wang).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.10.016

Therefore, the objectives of present study were to investigate the effects of various organic solvent stress treatments on the yields of EPSs by *B. licheniformis* OSTK95 and strain UD061. Besides, the antioxidant properties of the crude EPSs obtained from the culture were assessed using different in vitro systems. The results could revealed potential directions for further improvement of the producing strain for EPS by mutagenesis and offered a novel method for strain breeding.

2. Materials and methods

2.1. Microorganism and their cultivation

Wild-type strain OSTK95 excreted antioxidant EPSs, was isolated from marine mud, and later identified as *B. licheniformis* based on morphological, physiological, and biochemical properties. After UV and DES mutagenetic treatments, the high-yielding mutant UD59 with the productivity of the EPSs of 62.15 mg L^{-1} was screened from mutant library (date not shown). The strains were maintained on modified 2216E medium agar slants having composition (per liter aged seawater): 5 g tryptone, 1 g yeast extract, 0.1 g $FePO_4$, agar 2.0, and the medium were adjusted to pH 7.0. After incubating at 25 °C for 36 h, the slants were stored at 4 °C, and sub-cultured every 2 months.

2.2. Inoculum preparation and flask cultures

Seed cultures were prepared by transferring a loop of lawn from the fully grown slant into a 250 mL flask containing 50 mL of 2216E medium, and incubated at 25 °C on a rotary shaker at 200 rpm for 36 h. 0.5 mL of this precultured fluid was inoculated in 250 mL Erlenmeyer flask containing 50 mL of main culture medium with the following composition (per liter aged seawater): 10 g glucose, 5 g tryptone, 1 g yeast extract, 3 g KH_2PO_4, 1 g K_2HPO_4, 0.5 g $MgSO_4$. The initial pH of the medium was adjusted to 7.0 by 1 N NaOH or 1 N HCl. Main culture medium was maintained at the same conditions for 48 h.

2.3. Determination of growth curves of strains

Seed cultures were prepared by the method described above. Next, 1 mL of this pre-culture was used to inoculate a second culture of main culture medium (100 mL). The flask was incubated at 200 rpm, 25 °C for 38 h. Samples were collected at different time intervals during the fermentation to determine biomass concentration. Bacterial growth was determined by measuring the optical density at 600 nm at 2 h intervals. Growth rate determination was done in triplicate wells in three replicate experiments (Hassler et al., 2011).

2.4. Organic solvent tolerance of strain OSTK95 and strain UD059

The solvent tolerance of the microorganism was checked on liquid main culture medium. Strain OST23a and strain UD292 inoculated into the main culture medium respectively and then incubated at 25 °C and 200 rpm overnight. This pre-cultured fluid (0.5 mL) was inoculated in 250 mL Erlenmeyer flask containing 45 mL of main culture medium and 5 mL of organic solvent at 25 °C with shaking at 200 rpm. All flasks were plugged with chloroprene rubber stopper. After 48 h the cell growth was monitored by dry cell weight using the method reported before (Liu, Fang, et al., 2009).

2.5. Effect of different organic solvent stress on exopolysaccharidess production

To determine the effect of different organic solvent stress on the exopolysaccharidess production, the bacteria were cultured for 16 h, n-hexane were added to flask to 7.5%, 10%, 12.5%, 12.5% (v/v), while xylene were added to flask to 2.5%, 5%, 7.5% and 10% (v/v). Flasks were capped with chloroprene rubber stopper to prevent the volatilization of solvent and then cultured at 30 °C, 200 rpm for 3 h. Bacterial cells were harvested by centrifuging the culture medium at 12,000 × *g*, 4 °C for 10 min. The cell pellets, washed two times with sterile distilled water, was used to make seed cultures to produce EPSs with the media and cultural conditions mentioned above.

2.6. Determination of EPSs production

Samples collected from the fermentation broth was properly diluted and centrifuged at 8000 × *g* for 15 min. The resulting supernatant was filtered through a 0.45 μm membrane filter, mixed with four volumes of anhydrous ethanol, stirred vigorously and at 4 °C for further overnight. The precipitate from the ethanol dispersion was collected by centrifugation at 10,000 × *g* for 15 min, redissolved in distilled water and followed by deproteinization with 1/5 volume of Sevag reagent ($CHCl_3$–BuOH, v/v = 5/1) for seven times. The deproteinized solution was then dialyzed against distilled water, concentrated, lyophilized to afford the crude EPSs. Total EPSs concentrations in the ethanolic extracts were measured by using the phenol–sulfuric acid method (Ravella et al., 2010). The main values and the standard deviation were calculated from the data obtained with triplicate trials.

2.7. Assay of antioxidant activities in vitro of EPSs

2.7.1. Determination of reducing power of EPSs

The reducing power was determined according to the method of Li with some modification (Song et al., 2008). The reaction mixtures contained 2.5 mL phosphate buffer (pH 6.6, 0.2 M), 2.5 mL potassium ferricyanide (1%, w/v) and the EPS (5–250 mg L^{-1}). After incubating at 50 °C for 20 min, 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture for terminating the reaction, and then centrifuged at 5000 × *g* for 10 min. The supernatant (5 mL) was mixed with 0.5 mL ferric chloride (0.1%, w/v), and the absorbance was read spectrophotometrically at 700 nm. A higher absorbance indicates a higher reducing power. Deionized water and ascorbic acid were used as the blank and control, respectively.

2.7.2. Superoxide anion scavenging activity

The superoxide anion scavenging activity of EPS was determined according to the method of Stewar and Beewley with some modifications (Stewart & Bewley, 1980). The reaction mixture contained each 1.0 mL of NBT solution (156 μmol L^{-1} of NBT in 0.1 M phosphate buffer, pH 7.4), NADH solution (468 μmol L^{-1} of NADH in 0.1 M phosphate buffer, pH 7.4) and EPS solution (5–250 mg L^{-1}). After started the reaction mixture by adding 1.0 mL of PMS solution (60 μmol L^{-1} PMS in 0.1 M phosphate buffer, pH 7.4) at 25 °C for 5 min, the absorbance at 560 nm was measured. Deionized water and ascorbic acid BHT were used as the blank and control, respectively. The scavenging activity on superoxide radical (%) = $(1 - A_{sample}/A_{blank}) \times 100$.

2.7.3. Hydroxyl radical scavenging assay by EPS

The hydroxyl radical scavenging activity was measured by the method of Liu, Luo, et al. (2009). The reaction mixture contained 1 mL of 0.75 mM 1,10-phenanthroline, 1.5 mL of 0.15 M sodium phosphate buffer (pH 7.4), 1 mL of 0.75 mM FeSO4, 1 mL of H_2O_2

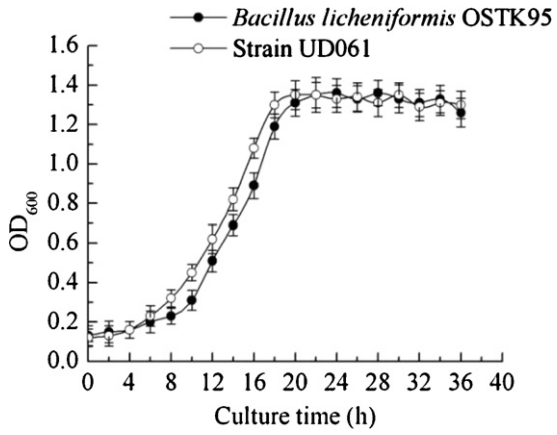

Fig. 1. Growth curve of *Bacillus licheniformis* OSTK95 and strain UD061.

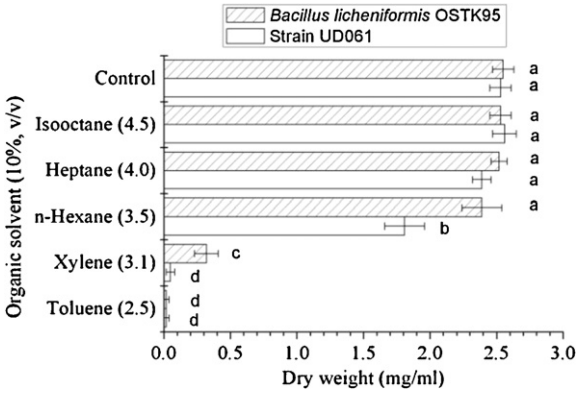

Fig. 2. Effects of organic solvents with different log *P* on the growth of *Bacillus licheniformis* OSTK95 and strain UD061. *Note*: The different letters mean significant difference at 0.05 levels.

(0.01%, v/v) and 0.5 mL of the EPS (5–250 mg L^{-1}). After incubating at 37 °C for 30 min, the absorbance of the EPS was measured at 560 nm. Deionized water and ascorbic acid were used as the blank and control, respectively. The scavenging activity on hydroxyl radical (%) = $(A_{sample} - A_{blank})/(A_0 - A_{blank}) \times 100$, where A_0 was the absorbance of the deionized water instead of H_2O_2 and sample in the assay system.

2.8. Genetic stability test

The genetic stability of the mutants, which is essential for the fermentation industry and also reflects the occurrence of mutations at the gene level, was examined by subculturing as described later. The mutant was streaked or spread and cultivated on the solid medium for *r* ten generations. Each generation was cultivated for 24 h at 25 °C. Ten colonies were selected randomly in each generation to produce the EPSs by fermentation.

3. Results and discussion

3.1. The growth curves of strains

Data in Fig. 1 represent the growth (A_{600}) curves of strain OSTK95 and strain UD061 during 36 h of incubation at 25 °C. The strains' growing pattern showed the growing curve were similar to "S' shape. The exponential (log) growth phase was reached between 6 and 18 h for *B. licheniformis* OSTK95 and between 8 and 20 h for strain UD061. The maximal OD (A_{600} nm) obtained for *B. licheniformis* OSTK95 and strain UD061 was ~1.35. The bacterial cells in mid-late of logarithmic growth phase are very sensitive to environmental factors. So the cells in this phase (16 h) were gathered for the research of next step in treatment with organic solvent (Freitas, Alves, & Reis, 2011).

3.2. Organic solvent tolerance of strain OST23a and strain UD292

The growth of the strain *B. licheniformis* OSTK95 and strain UD292 in the liquid medium containing various organic solvents was studied. Fig. 2 shows the effect of the organic solvents on the dry cell weights of these strains after 48 h of cultivation. *B. licheniformis* OSTK95 did not grow in the presence of the organic solvent with log *P* values below 3.5. While strain UD292 grew on the medium with 10% (v/v) organic solvent with log *P* values equal to or above 3.1. Organic solvents are known to be extremely toxic to cells. They dissolve and accumulate in the bacterial cell membrane, resulting in changes in structural and functional integrity and cause cell lysis. The parameter log *P* of organic solvent is defined as the partition coefficient of the given solvent in an equimolar mixture of octanol and water. The greater the polarity, the lower the log *P* value and the greater the toxicity of the solvent (Heipieper, Neumann, Cornelissen, & Meinhardt, 2007). A polysaccharide layer coupled with biofilm production protects bacterial cells from organic compounds and enhances the possibility of colonization in special ecological niches (Hung, Santschi, & Gillow, 2005; Onbasli & Aslim, 2009). This may be the reason of the interesting phenomenon that strain UD061 can grow on the medium with 10% (v/v) organic solvent with log *P* values equal to 3.1 but *B. licheniformis* OSTK95 cannot.

3.3. Effect of different organic solvent stress on exopolysaccharidess production

Improved production of exopolysaccharidess of *B. licheniformis* OSTK95 and strain UD061 by different concentration of different organic solvent stress treatment is shown in Figs. 3–5. In general, the yield of exopolysaccharides increased with higher concentrations of organic solvent. The maximum productivity of the EPS of *B. licheniformis* OSTK95 was 68.59 mg L^{-1} after treated with 10% n-hexane or 1% xylene, while the maximum productivity of the EPS of strain UD061 was 185.01 mg L^{-1} after treatment with 12.5% n-hexane or 5% xylene. The possible reason is that *B. licheniformis* OSTK95 is more sensitive than strain UD061 to the organic solvent with the same log *P*. It is interesting that organic solvent stresses not only improve the yield of exopolysaccharidess of wild type strain

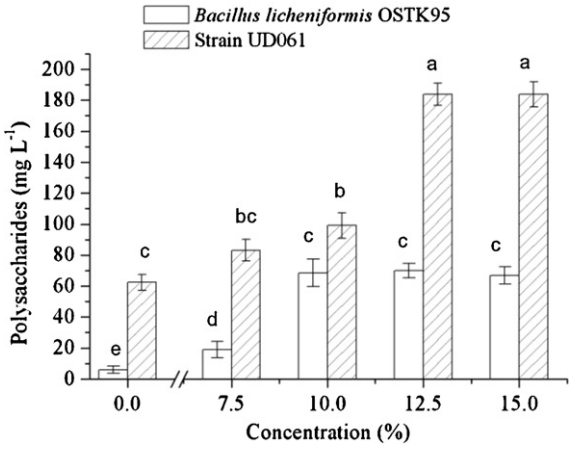

Fig. 3. Effects of different levels of n-hexane on EPSs production of *Bacillus licheniformis* OSTK95 and strain UD061. *Note*: The different letters mean significant difference at 0.05 levels.

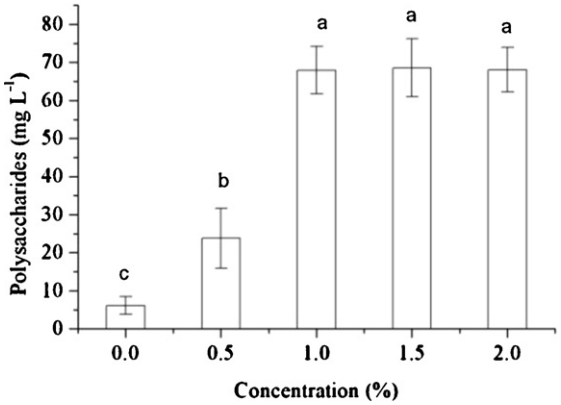

Fig. 4. Effects of different levels of xylene on EPSs production of *Bacillus licheniformis* OSTK95. *Note*: The different letters mean significant difference at 0.05 levels.

but also improve that of mutant, which indicated that an abnormal mechanism in *B. licheniformis* was induced by organic solvent stress.

The same result was reported that *Pseudomonas aeruginosa* B1, *P. fluorescens* B5, *P. stutzeri* B11 and *P. putida* B15 had exhibited high production of EPSs in presence of various organic pollutants (2,4-D, benzene, BTX and gasoline, respectively) (Onbasli & Aslim, 2009). Polysaccharides are believed to protect bacterial cells from organic compounds thus to enhance the chances of the cells to colonize special ecological niches (Freitas et al., 2011; Gonzalez et al., 2010; Kang & Park, 2010). But adding organic solvent to the media would cause environmental pollution and would harm and danger the people's health (Ta-Chen, Chang, & Young, 2008). So organic solvent stress treatment but not adding organic solvent to the media was used to improve the production of exopolysaccharidess of *B. licheniformis*. Organic solvent stress treatment would be a new effective method for induced mutation breeding of strain produce exopolysaccharidess and deserves further research in the future.

3.4. The antioxidant activities in vitro of EPSs

Antioxidant activity is one of the main characters of exopolysaccharidess from *B. licheniformis*. The antioxidant activities of the exopolysaccharidess from the wild-type strain OSTK95 and mutant strain UD061 before or after treatment were compared and the results indicated that the antioxidant activities did not change significantly (Table 1).

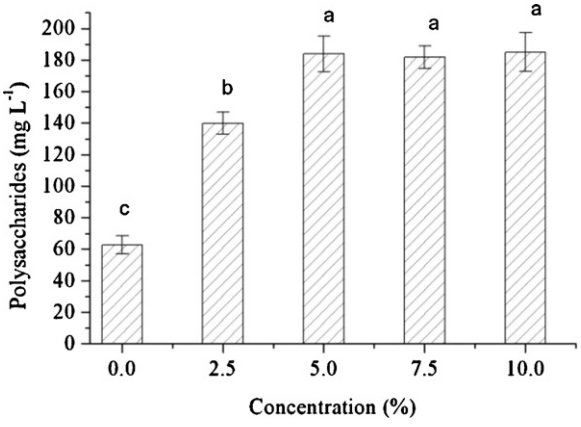

Fig. 5. Effects of different levels of xylene on EPSs production of strain UD061. *Note*: The different letters mean significant difference at 0.05 levels.

Table 1
Antioxidant activities in vitro of EPS of different strains.

Strain	Reducing power (A_{700})	Superoxide anion scavenging (%)	Hydroxyl radical scavenging (%)
OSTK95^{w}	0.31 ± 0.02	43.89 ± 3.65	50.91 ± 4.33
OSTK95^{s}	0.33 ± 0.03	41.52 ± 3.12	52.09 ± 3.56
UD061^{w}	0.32 ± 0.05	42.09 ± 3.18	51.95 ± 4.56
UD061^{s}	0.30 ± 0.02	42.11 ± 3.02	50.95 ± 3.06

Note: w, wild type strain; s, strain treated by organic solvent.

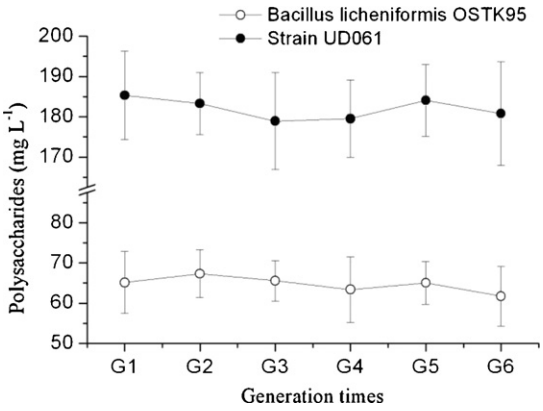

Fig. 6. Tests for hereditary stability of *Bacillus licheniformis* OSTK95 and strain UD061.

3.5. Genetic stability test

B. licheniformis OSTK95 and strain UD292 were generated and inoculated into flask fermentations to test its stability in EPS yield (Fig. 6). It can be seen from the results that EPSs yield remained relatively stable in subsequent generations, which suggested that the change was due to the inheritable mutation induced by organic solvent stress treatment.

4. Conclusions

A novel method for improving the production of antioxidant exopolysaccharidess from microorganism was described. The concentration of n-hexane and xylene stress treatment affects the yield of exopolysaccharides produced by *B. licheniformis* OSTK95 and strain UD061. The study has also confirmed that the effects of organic solvent stress treatment on the concentration of EPS produced by *B. licheniformis* OSTK95 and strain UD061 was due to the inheritable mutation. Further work on the molecular mechanism of the novel method is in progress.

References

Fang, Y., Lu, M., Xu, W., Liu, S., Jiao, Y., & Wang, S. (2010). Screening and optimization of fermentation medium of an antioxidant exopolysaccharides-producing marine strain. *Food Science*, *31*(23), 5.

Freitas, F., Alves, V. D., & Reis, M. A. (2011). Advances in bacterial exopolysaccharidess: From production to biotechnological applications. *Trends in Biotechnology*, *29*(8), 388–398.

Gonzalez, A. G., Shirokova, L. S., Pokrovsky, O. S., Emnova, E. E., Martinez, R. E., Santana-Casiano, J. M., et al. (2010). Adsorption of copper on *Pseudomonas aureofaciens*: Protective role of surface exopolysaccharidess. *Journal of Colloid and Interface Science*, *350*(1), 305–314.

Harrah, T., Panilaitis, B., & Kaplan, D. (2007). Microbial exopolysaccharidess, The Prokaryotes, 766–776.

Hassler, C. S., Alasonati, E., Mancuso Nichols, C. A., & Slaveykova, V. I. (2011). Exopolysaccharidess produced by bacteria isolated from the pelagic Southern Ocean – Role in Fe binding, chemical reactivity, and bioavailability. *Marine Chemistry*, *123*(1–4), 88–98.

Heipieper, H. J., Neumann, G., Cornelissen, S., & Meinhardt, F. (2007). Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. *Applied Microbiology and Biotechnology*, *74*(5), 961–973.

Hung, C.-C., Santschi, P. H., & Gillow, J. B. (2005). Isolation and characterization of extracellular polysaccharides produced by *Pseudomonas fluorescens* Biovar II. *Carbohydrate Polymers*, *61*(2), 141–147.

Kang, Y. S., & Park, W. (2010). Protection against diesel oil toxicity by sodium chloride-induced exopolysaccharidess in *Acinetobacter* sp. strain DR1. *Journal of Bioscience and Bioengineering*, *109*(2), 118–123.

Kuzma, M., Clack, B., Edwards, J., Tylingo, R., Samaszko, J., & Madaj, J. (2012). Structure and properties of the exopolysaccharidess produced by *Pseudomonas mutabilis* T6 and *P. mutabilis* ATCC 31014. *Carbohydrate Research*, *348*, 84–90.

Lima, L. F., Habu, S., Gern, J. C., Nascimento, B. M., Parada, J. L., Noseda, M. D., et al. (2008). Production and characterization of the exopolysaccharidess produced by *Agaricus brasiliensis* in submerged fermentation. *Applied Biochemistry and Biotechnology*, *151*(2–3), 283–294.

Lin, E.-S. (2010). Production of exopolysaccharidess by submerged mycelial culture of Grifola frondosa TFRI1073 and their antioxidant and antiproliferative activities. *World Journal of Microbiology and Biotechnology*, *27*(3), 555–561.

Liu, J., Luo, J., Ye, H., Sun, Y., Lu, Z., & Zeng, X. (2009). Production, characterization and antioxidant activities in vitro of exopolysaccharidess from endophytic bacterium *Paenibacillus polymyxa* EJS-3. *Carbohydrate Polymers*, *78*(2), 275–281.

Liu, S., Fang, Y., Xu, W., Lu, M., Wang, S., & Chen, L. (2009). Screening and identification of a novel organic solvent-stable lipase producer. *Annals of Microbiology*, *59*(3), 539–543.

Liu, J., Luo, J., Ye, H., Sun, Y., Lu, Z., & Zeng, X. (2010). Medium optimization and structural characterization of exopolysaccharidess from endophytic bacterium *Paenibacillus polymyxa* EJS-3. *Carbohydrate Polymers*, *79*(1), 206–213.

Luo, J., Liu, J., Ke, C., Qiao, D., Ye, H., Sun, Y., et al. (2009). Optimization of medium composition for the production of exopolysaccharidess from *Phellinus baumii* Pilát in submerged culture and the immuno-stimulating activity of exopolysaccharidess. *Carbohydrate Polymers*, *78*(3), 409–415.

Onbasli, D., & Aslim, B. (2009). Effects of some organic pollutants on the exopolysaccharidess (EPSs) produced by some *Pseudomonas* spp. strains. *Journal of Hazardous Materials*, *168*(1), 64–67.

Ravella, S. R., Quiñones, T. S., Retter, A., Heiermann, M., Amon, T., & Hobbs, P. J. (2010). Extracellular polysaccharide (EPS) production by a novel strain of yeast-like fungus *Aureobasidium pullulans*. *Carbohydrate Polymers*, *82*(3), 728–732.

Satpute, S. K., Banat, I. M., Dhakephalkar, P. K., Banpurkar, A. G., & Chopade, B. A. (2010). Biosurfactants, bioemulsifiers and exopolysaccharidess from marine microorganisms. *Biotechnology Advances*, *28*(4), 436–450.

Song, L., Li, T., Yu, R., Yan, C., Ren, S., & Zhao, Y. (2008). Antioxidant activities of hydrolysates of arca subcrenata prepared with three proteases. *Marine Drugs*, *6*(4), 607–619.

Stewart, R. R. C., & Bewley, J. D. (1980). Lipid peroxidation associated with accelerated aging of soybean axes. *Plant Physiology*, *65*(2), 245–248.

Ta-Chen, L., Chang, J. S., & Young, C. C. (2008). Exopolysaccharidess produced by Gordonia alkanivorans enhance bacterial degradation activity for diesel. *Biotechnology Letters*, *30*(7), 1201–1206.

Wang, C. L., Huang, T. H., Liang, T. W., Fang, C. Y., & Wang, S. L. (2011). Production and characterization of exopolysaccharidess and antioxidant from *Paenibacillus* sp. TKU023. *New Biotechnology*, *28*(6), 559–565.