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EXPERIMENTAL ARTICLES

Potential Probiotics of the Far Eastern Trepang *Apostychopus japonicus* Producing Digestive Enzymes

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Abstract—Data were obtained on species diversity of the digestive tract microflora of the Far Eastern trepang *Apostychopus japonicus*. The probiotic characteristics of microbial isolates were investigated related to their capacity for synthesis of digestive enzymes (amylase, chondroitin sulfatase, chitinase, and alginate lyase). The *Pseudomonas stutzeri* strain exhibiting high activity of all the investigated enzymes was found to be preferable among the potential probiotics used for cultivation of the Far Eastern trepang. Preparations based on mixed cultures of a *Bacillus pumilus* strain with high chondroitin sulfatase and chitinase activities with strains of *B. coagulans* and *B. megaterium* K13 with high activity of amylases and alginate lyases are also promising.

Key words: Far Eastern trepang *Apstychopus japonicus*, probiotics, amylase, chondroitin sulfatase, chitinase, alginate lyase.

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Due to intense fishing unobservant of fishing regulations, the numbers of the Far Eastern trepang *Apostychopus japonicus* (Selenka, 1867) have decreased significantly throughout its range. Since the numbers of these invertebrates cannot be recovered by natural reproduction, maricultural cultivation is evidently necessary [1].

In spite of the obvious advantages of marine farms, decreased immunity of hydrobionts and susceptibility to diseases are common under such conditions, resulting from continuous presence of stress factors (high biomass load per volume, organic contamination of the medium, and gradients of oxygen concentration) [2]. Application of antibiotics is not always efficient and results in deficiencies of microorganisms, including those involved in food digestion. The application of preparations based on probiotics, i.e., living microorganisms and/or compounds of microbial or other origin that exhibit a favorable effect on the physiological functions, biochemical, and behavioral reactions of the host organisms by optimization of its microbial status is among the possible approaches to health preservation and number recovery of the Far Eastern trepang [3]. Several mechanisms of the favorable effect of probiotics on the organisms are known, including the synthesis of digestive enzymes that improve the activity of the gastrointestinal tract [4]. The organisms involved in such symbiotic relations with hydrobionts may facilitate digestion of such difficultly assimilated natural polymers as chitin, alginates, chondroitin sulfate, etc., which arrive at the trepang's digestive tract with sediments and debris of marine animals and algae.

Data on the qualitative composition of the microflora of the Far Eastern trepang are presently scarce [5], while information concerning the enzymes synthesized by the holothurians' microflora is completely absent.

The goal of the present work was to isolate the strains of the trepang microflora that exhibit high activities of digestive enzymes (amylase, chitinase, chondroitin sulfatase, and alginate lyase) for subsequent application as probiotics for the cultivation of hydrobionts under industrial conditions.

MATERIALS AND METHODS

Isolation and identification of the microflora of the Far Eastern trepang. For investigation of the microflora of the Far Eastern trepang (*Apostychopus japonicus*), in 2004 and 2006 adult specimen were collected from the coastal zone of the Popov Island (five) and the Kievka Bay in the Sea of Japan, (Primorskii krai, Russia) (eight). The intestines of the trepangs were aseptically removed and placed into individual penicillin vials with liquid CMM medium (medium for marine microorganisms) [6]. Enrichment cultures were obtained by 24-h incubation at 30°C; the material was then spread-inoculated on CMM agar and incubated for two days under the same conditions. Material from morphologically different isolated colonies was then transferred to slanted CMM agar with

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a microbiological loop. The enzymatic activity of pure cultures was determined by inoculation on solid and liquid media with chitin [7], sodium alginate [8], starch, and chondroitin sulfate [9]. The strain exhibiting activities on these media were identified using the API biochemical tests (bioMerieux, France).

Determination of amylase and chondroitin sulfatase activities. For qualitative assessment of amylolytic and chondroitin sulfatase activity, the strains were inoculated on plates with mineral–salt agar media with starch and chondroitin sulfate, respectively. The cultivation was carried out for three days at 30°C. Lugol's solution was then poured over the plates. On starch media, transparent zones were visualized around the colonies; on chondroitin sulfate media, the zones of substrate hydrolysis around the colonies were revealed.

For qualitative determination of enzyme productivity, 24-h cultures of the isolates exhibiting activity on solid media were used to prepare suspensions (2×10^9 cells/ml), which were resuspended in CMM medium and incubated on a shaker at 30°C for two days. The enzymatic activity was determined by the Somogyi method [10]. An aliquot of the culture liquid (0.5 ml) obtained by 5-min centrifugation at 10000 *g* was supplemented with 4.5 ml of the substrate (1 g starch or 1 mg chondroitin sulfate per 100 ml buffer for respective enzymatic activities). In the control, distilled water was used instead of the culture liquid. After shaking for 30 min at 37°C, the samples were boiled for 5–10 min. The solution (1 ml) was supplemented with a 1 ml of solution A (Na_2CO_3, 24 g; H_2O, 200 ml; $NaHCO_3$, 16 g; H_2O, 200 ml; $CuSO_4$, 4 g; H_2O, 200 ml; Na_2SO_4, 180 g), and the test tubes were boiled in a water bath for 20 min. After cooling, 1 ml of solution B ($(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$, 25 g; H_2O, 450 ml; H_2SO_4, 22 ml; Na_2HAsO_4, 3 g; H_2O, 25 ml) was added. The stain developed after 1 min; the samples were then supplemented with 7 ml of distilled water and OD_{620} was determined. The amount of enzymes forming 1 μmol/ml glucose during 1 min under experimental conditions was accepted as a respective unit of amylase and chondroitin sulfatase activity.

Determination of chitinolytic activity. Chitinolytic activity was assayed in dynamics (after 2, 4, 7, and 9 days) as zones of hydrolysis on agar medium with chitin as the only source of carbon and nitrogen.

For further investigation of bacteria exhibiting activity on solid media, bacterial suspensions (2×10^9 cells/ml) of 24-h cultures were inoculated to liquid medium with native chitin (10 g/l) and incubated on a shaker for 9 days [7]. Chitinolytic activity was determined in dynamics after 2, 4, 7, and 9 days by the modified colorimetric method of *N*-acetylaminosugar determination [11]. The optical density of the media was determined spectrophotometrically at 582 nm. The content of reducing sugars was calculated from the calibration curve obtained with *N*-acetyl-D-glucosamine as a standard. Chitinase activity was expressed in 1-mg reducing sugars formed by hydrolysis of colloidal chitin with 1 ml of the culture liquid during 1 h at 37°C (mg/(ml h)).

Determination of alginate lyase activity. For qualitative assessment of alginate lyase activity, agarized medium with sodium alginate was used and substrate hydrolysis around the colonies was determined 2, 3, and 4 days after inoculation. Bacterial suspensions (2×10^9 cells/ml) prepared from 24-h cultures exhibiting activity on solid media were used to inoculate liquid medium with sodium alginate (10 g/l); the cultures were then incubated on a shaker for 4 days at 30°C [8]. The culture liquid was obtained after centrifugation, and the enzymatic activity was determined by the viscosimetric method, as changes in viscosity of a 0.3% sodium alginate solution in Tris–HCl buffer with 1.9% NaCl. The reaction mixture contained 10 ml of sodium alginate solution and 1 ml of the culture liquid. The mixture containing 1 ml of the buffer instead of the culture liquid was used as the control. The measurements were carried out with an Ostwald viscosimeter. Changes in viscosity were determined after 20 min of enzymatic action at 37°C. The decrease in relative viscosity was calculated from the equation:

$$\eta = (t_k - t_0)/t_k \times 100\%,$$

where t_k is the flow time for the control solution (10-ml sodium alginate and 1-ml buffer solution) and t_0 is the flow time for the experimental solution.

RESULTS AND DISCUSSION

Isolation and identification of the trepang microflora. From the intestines of Far Eastern trepangs collected at the coastal zone of the Popov Island and the Kievka Bay, a total of 67 strains were isolated (26 and 41, respectively) and used to determine their capacity for decomposition of starch, chondroitin sulfate, chitin, and sodium alginate. The strains able to synthesize the relevant enzymes were identified by biochemical methods.

Amylase activity. Numerous species of marine bacteria are able to degrade starch; their activity is usually within the range of 0.001–1.343 U (μmol/(ml min)) [12]. Among our 67 isolates, only 8 (about 12%) exhibited a degree of enzymatic activity (Table 1). Among the strains from the intestines of the Popov Island trepangs, the strains of *Aeromonas hydrophila* and *Pseudomonas stutzeri* had the highest capacity for starch hydrolysis, exhibiting both good growth on solid media (8- and 10-mm hydrolysis zones) and high amylase activities (0.382 and 0.337 U, respectively). The strain of *Enterobacter intermedius* hydrolyzed the substrate relatively weakly (4 mm) and had low enzyme activity (0.046 U).

Among the strains from the intestines of the Kievka Bay trepangs, the strains of *Bacillus coagulans* and *B. megaterium* K13 exhibited the most active starch hydrolysis, with amylase activity of 0.587 and 0.593 U, respectively. High levels of starch decomposition were

Table 1. Quantitative assessment of amylolytic activity of the trepang microflora from the coastal waters of Popov Island and Kievka Bay

Bacterial species	Zone of substrate hydrolysis around the colonies, mm	Amylase activity, U (μmol/(ml min))
Popov Island		
Aeromonas hydrophila	10	0.382 ± 0.021
Enterobacter intermedius	4	0.046 ± 0.009
Pseudomonas stutzeri	8	0.337 ± 0.011
Kievka Bay		
Bacillus coagulans	10	0.587 ± 0.016
B. megaterium K13	12	0.593 ± 0.024
B. subtilis	7	0.327 ± 0.031
B. megaterium K20	9	0.455 ± 0.027
B. circulans	8	0.441 ± 0.021

Table 2. Quantitative assessment of chondroitin sulfatase activity of bacteria isolated from the Far Eastern trepang (Popov Island)

Bacterial species	Zone of substrate hydrolysis around the colonies, mm	Chondroitin sulfatase activity, U (mmol/(μl min))
Pseudomonas stutzeri	6	0.387 ± 0.012
P. putida	4	0.146 ± 0.016
P. fluorescens	4	0.124 ± 0.026
Bacillus pumilus	8	0.412 ± 0.032

also found in three other strains with amylase activities of 0.327–0.455 U, in accordance with the results obtained on solid media (7- to 9-mm hydrolysis zones). These higher levels of enzyme activity are probably not accidental and result rather from the higher carbohydrate content in the silt–sandy sediments of Kievka Bay compared to the stone–sandy sediments of Popov Island [13]. These characteristics of the environment result in intense development of specific microflora capable of active starch decomposition on the surface of silt–sandy sediments and, therefore, in the intestines of detritus consumers.

Chondroitin sulfatase activity. Only four strains of our collection (about 6%) isolated from the Popov Island trepangs were able to grow on the medium with chondroitin sulfate: *P. stutzeri, P. putida, P. fluorescens*, and *B. pumilus* (Table 2).

Table 2 demonstrates that the strains of *B. pumilus* and *P. stutzeri* exhibited high enzymatic activity (0.412 and 0.387 U, respectively) and were able to efficiently decompose the substrate, in accordance with the results obtained on solid media. Production of extracellular chondroitin sulfatase by the strains of *P. putida* and *P. fluorescens* was relatively weak (activity of 0.146 and 0.124 U, respectively); this result explains the low degrees of chondroitin sulfate hydrolysis on solid media.

According to the literature, chondroitin sulfatase activity in the range from 0.001 to 0.643 U is usually found in bacteria associated with benthic vertebrates and invertebrates [12]. In our study, the enzymatic activity of bacteria isolated from the holothurians was 0.124–0.412 U, i.e., these microorganisms exhibited relatively high capacity for hydrolysis and assimilation of chondroitin sulfates, a component of vertebrate connective tissue.

None of the strains isolated from the Kievka Bay holothurians possessed chondroitin sulfatase activity. This may be explained by the presence of endogenous enzymes in the animals. While detritus of animal origin is known to be efficiently utilized by the trepang [14], the nature of the enzymes involved in this process remains unknown. The intestinal cells of the trepangs from both sites are probably able to produce chondroitin sulfatase; in the Popov Island holothurians, however, enzymes of bacterial origin contribute significantly to assimilation of detritus of animal origin.

Chitinase activity. Chitinase activity was assayed in dynamics (after 2, 4, 7, and 9 days) as hydrolysis zones around the colonies on agar media with colloidal chitin as the only source of carbon and nitrogen (Table 3).

Only four strains (about 6% of the collection) from the Popov Island trepangs exhibited some degree of chitin hydrolysis around the colonies. Similarly to

Table 3. Quantitative assessment of chitinolytic activity of bacteria isolated from intestines of the trepang *Apostychopus japonicus* (Popov Island)

Bacterial species	Zone of substrate hydrolysis around the colonies, mm				Chitinolytic activity, U (mg/(ml min))			
	day 2	day 4	day 7	day 9	day 2	day 4	day 7	day 9
Enterobacter hormaechei	1	**3**	3	3	0.15 ± 0.02	0.27 ± 0.07	**0.29 ± 0.01**	0.12 ± 0.02
Pseudomonas stutzeri	4	6	**8**	8	1.13 ± 0.06	1.78 ± 0.02	**2.09 ± 0.05**	1.71 ± 0.05
Bacillus pumilus	1	**3**	3	3	0.02 ± 0.01	0.56 ± 0.02	**0.87 ± 0.03**	0.61 ± 0.01
P. fluorescens	1	**3**	3	3	0.51 ± 0.04	**0.64 ± 0.01**	0.55 ± 0.05	0.38 ± 0.02

Table 4. Quantitative assessment of alginate lyase activity of the trepang microflora

Bacterial species	Zone of substrate hydrolysis around the colonies, mm			Viscosity decrease, %		
	day 2	day 3	day 4	day 2	day 3	day 4
Pseudomonas stutzeri Popov Island	3	**6**	6	55.3 ± 0.3	**75.4 ± 0.7**	75.1 ± 0.7
P. fluorescens Popov Island	2	**4**	4	24.1 ± 0.2	**29.7 ± 0.2**	29.4 ± 0.2
Bacillus coagulans Kievka Bay	4	**8**	8	45.2 ± 0.8	**78.1 ± 0.5**	78.3 ± 0.5
B. megaterium K13 Kievka Bay	2	**7**	7	31.4 ± 0.3	**73.6 ± 0.4**	73.4 ± 0.2
B. subtilis Kievka Bay	0	**3**	3	16.7 ± 0.6	**31.2 ± 0.4**	30.1 ± 0.4

chondroitin sulfatase activity, the absence of chitinases in bacteria from the Kievka Bay holothurians may result from the presence of endogenous enzymes in the trepangs from this site.

Although the mentioned bacterial strains exhibited activity on the second day of cultivation, the highest chitinase synthesis was observed on the fourth and seventh days, similarly to the results of other authors [15, 16]. The most pronounced hydrolysis zones were observed around *P. stutzeri* colonies (4–8 mm); in the case of other strains, activity was relatively low (1–3 mm) and decreased after 4 days.

For quantitative determination of chitinase activity in dynamics (2, 4, 7, and 9 days), 24-h cultures grown on solid medium were inoculated into the medium with ground native chitin. Activity was determined as the amount of reducing sugars resulting from hydrolysis of colloidal chitin using the colorimetric reaction with 3,5-dinitrosalycilic acid. The results are presented in Table 3.

The ability to produce chitinases is known to occur primarily in members of the genera *Aeromonas, Bacillus, Pseudomonas, Vibrio, Clostridium, Serratia*, etc. Microbial chitinase activity, depending on the source of isolation and individual characteristics of microorganisms, may vary from 0.015 to 2.4 U [7, 17–20]. The highest enzyme activity (1.72–2.4 U) was observed in bacteria isolated from the gastrointestinal tract of marine fish and invertebrates.

Our investigation demonstrated that, in the case of *P. fluorescens*, activity peaked on the fourth day (0.64 U) and was significantly lower on the seventh day (0.55 U). For other strains, the content of reducing sugars peaked on the seventh day and decreased on the ninth day of the experiment. The *P. stutzeri* strain produced the highest amount of reducing sugars (1.13–2.09 U); this is in accordance with the results obtained on agar medium with chitin (hydrolysis zones up to 8 mm). The *Enterobacter hormaechei* strain exhibited low activity both on solid (1–3 mm) and in liquid medium (0.12–0.29 U).

Alginate lyase activity. Qualitative assessment of alginate lyase activity was carried out on agar medium with sodium alginate by monitoring substrate hydrolysis around the colonies after 2, 3, and 4 days. The results are presented in Table 4.

Out of 67 strains, only 7% exhibited alginate lyase activity, two from the Popov Island trepangs (*P. stutzeri* and *P. fluorescens*) and three from the Kievka Bay (*B. coagulans, B. megaterium* K13, and *B. subtilis*). In all those strains, the enzymatic activity was highest on the third day (hydrolysis zones of 3–8 mm). No qualitative changes in activity were observed on plates on the fourth and subsequent days of incubation.

Quantitative assessment of alginate lyase activity was carried out by the viscosimetric method by a decrease in the viscosity of 0.3% sodium alginate solution. Bacteria carried out the most efficient decrease in the viscosity of the test solution on the third day, in accordance with the results obtained on solid medium (Table 4).

Of five strains, *P. stutzeri, B. coagulans*, and *B. megaterium* K13 exhibited the highest levels of alginate lyase activity (75.4, 78.1, and 73.6%, respectively). The other two strains (*P. fluorescens* and *B. subtilis*) were significantly less efficient (29.7 and 31.2%).

It is known from the literature that alginate lyase activity occurs mostly in bacterial commensals, rather than in free-living microorganisms. According to Vydryakova et al. [18], the *Vibrio harvei* strains isolated from fish intestines and algal surface exhibited the highest efficiency (viscosity decrease by over 70%), while the strains from seawater were the least efficient (viscosity decrease by less than 5%). This was an indication of involvement of luminous bacteria *V. fisheri* in the transformation of plant biomass in deep seas, especially of their participation in plant biomass processing in the gastrointestinal tract of marine organisms [18].

Comparison of our results with the published data suggested that the isolated strains play an important part in processing and assimilation of vegetable food in the trepang organism. We believe that the strains of *P. stutzeri, B. coagulans*, and *B. megaterium* K13, which exhibited the highest values of enzymatic activity, are the most promising as potential probiotics.

Thus, for the first time, the present work provided information on the characteristics of the hydrolytic enzymes of bacteria isolated from the Far Eastern trepang *Apostychopus japonicus*. Of 67 strains, only *P. stutzeri* exhibited activity of all the four investigated enzymes. High activity of *B. pumilus* in hydrolysis of chondroitin sulfate and chitin should be mentioned, as well as of *B. coagulans* and *B. megaterium* K13 in respect to starch and sodium alginate.

The materials presented in this work may be useful both for further basic research in this field and for practical applications in the mariculture of hydrobionts.

REFERENCES

1. Sergeenko, V.A. and Dubrovskii, S.V., Effect of Fishery on the State of Far Eastern Trepang *Stichopus japonicus* Population in the Busse Lagoon (Sakhalin), in *Ekosistemy morei rossii v usloviyakh antropogennogo faktora. Tez. Dokladov Vserossiiskoi Nauchnoi Konferentsii g. Astrakhan'* (Ecosystems of Russian Seas Affected by Anthropogenic Factors), KaspNIRKh, 1994, pp. 65–73.
2. Burlachenko, I.V., Sudakova, N.V., Balakirev, E.I., Mordovtsev, D.A., and Malik, E.V., Promising Probiotics for Sturgeons, *Rybnoe Khozyaistvo*, 2006, no. 3, pp. 12–16.
3. Ursova, N.I., Prospects for Pediatric Application of Metabolite Type Probiotics, *Zhurn. Consilium-Medicum*, 2003, vol. 5, no. 6, pp. 3–6.
4. Verschuere, L., Heang, H., Criel, G., Dafnis, S., Sorgeloos, P., and Verstraete, W., Protection of *Artemia* against the Pathogenic Effects of *Vibrio proteolyticus* CW8T2 by Selected Bacterial Strains, *Appl. Environ. Microbiol.*, 2000, vol. 66, pp. 1139–1146.
5. Shul'gina, L.V., Microflora of the Trepang *Stichopus japonicus* Selenka Intestine, Seawater, and Detritus in the Peter the Great Bay, in *Ekologiya i usloviya vosproizvodstva ryb i bespozvonochnykh* (Ecology and Reproduction of Fish and Invertebrates), Vladivostok, 1982, pp. 152–156.
6. Youchimizu, M. and Kimura, T., Study of Intestinal Microflora of Salmonids, *Fish Pathol.*, 1976, vol. 10, no. 2, pp. 243–259.
7. Slabospitskaya, A.T. and Krymovskaya, S.S., Chitinases of Aerobic Spore-Forming Bacteria Isolated from Diverse Ecological Sources, *Mikrobiol Zhurn.*, 1992, vol. 54, no. 6, pp. 16–22.
8. Kitamikado, M., Yamaguchi, K., Tseng, C.H., and Okabe, B.I., Method of Alginatelyase Activity Determination, *Appl. Environ. Microbiol.*, 1990, vol. 56, pp. 2939–2940.
9. Wang, Y. and Xu, Z., Effect of Probiotics for Common Carp (*Cyprinus carpio*) Based on Growth Performance and Digestive Enzyme Activities, *Aquaculture*, 2005, vol. 43, pp. 32–35.
10. Somogyi, M., Notes on Sugar Determination, *J. Biol. Chem.*, 1952, vol. 195, pp. 19–23.
11. Reissing, J.L., Strominger, J.L., and Leloir, LF., Sugar Determination, *J. Biol. Chem.*, 1955, vol. 217, pp. 959–966.
12. Wonger, B., Amylase and Chondroitynsulfatase Activity of *Vibrio* sp., *Aquaculture*, 2003, vol. 22, pp. 53–58.
13. Khudyakova, Yu.V., Pivkin, M.V., Kuznetsova, T.A., and Svetashev, V.I., Fungi in Sediments of the Sea of Japan and Their Biologically Active Metabolites, *Mikrobilogiya*, 2000, vol. 69, no. 6, pp. 722–726 [*Microbiology* (Engl. Transl.), vol. 69, no. 6, pp. 608–611].
14. Levin, V.S., *Dal'nevostochnyi trepang* (Far Eastern Trepang), Vladivostok: Dal'nevost. knizhnoe izd-vo, 1982.
15. Sundarray, N. and Bhat, J.V., Breakdown of Chitin by *Cytophaga johnsonii*, *Arch. Microbiol.*, 1972, vol. 85, pp. 159–167.
16. Cody, R.M., Distribution of Chitinase and Chitobiase in *Bacillus, Curr. Microbiol.*, 1989, vol. 19, pp. 201–205.
17. Chigaleichik, A.G. and Pirieva, D.A., Extracellular Chitinase of *Aeromonas liquefaciens, Prikl. Biokhimiya Microbiologiya*, 1976, vol. 12, no. 2, pp. 238–242.
18. Vydryakova, G.A., Chugaeva, Yu.V., and Tyul'kova, N.A., Transformation of Biopolymers by Luminescent Bacteria, *Sib. Ekol. Zh.*, 2002, no. 2, pp. 137–144.
19. Ohtahara, A., Mitsutomi, M., and Uchida, Y., Purification and Some Properties of Chitinase from *Vibrio* sp., *J. Ferment. Technol.*, 1979, vol. 51, pp. 169–177.
20. Bennet, C.B. and Hood, M.A., Effects of Cultural Conditions on the Modulation of Chitinase by *Bacillus megaterium, Dev. Ind. Microbiol.*, 1980, vol. 21, pp. 357–363.