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Potential Use of the Phototrophic Bacterium, *Rhodopseudomonas palustris*, as an Aquaculture Feed

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Abstract

An indigenous strain of the phototrophic bacterium, *Rhodopseudomonas palustris*, was mass cultured in sago wastewater under anaerobic-light conditions. The bacterial cell mass was analyzed for nutritional value and tested for toxicity and acceptability as an aquaculture feed. Proximate analysis indicates a macrocomposition of 40% crude protein, 0.64% crude lipid and 2.09% crude fibre. The essential amino acids (EAA) of the bacteria biomass comprise 53% of its true protein content, and all EAA present were found to be within the dietary requirements of penaeid shrimps at the 35-55% dietary protein level. Ex-perimental feeding trials carried out on brine shrimp (*Artemia*) larvae showed that the bacteria, whether given as a sole diet or as a feed supplement, supported larval survival (42-53%) and growth (78-88%) with no significant differences. Larval survival of brine shrimps fed on bacteria or bacteria-supplemented diets (42-53% survival) were also compa-rable to those fed on a bacteria-free mixed diet of commercial prawn feed and the blue-green algae *Spirulina* (60%). However, after 7 days of rearing, brine shrimps fed the lat-ter mixed diet were significantly larger (121% increase in mean body length) than those fed the bacteria or bacteria-supplemented diets (78-88%).

Introduction

In shrimp larviculture, a variety of live foods, namely, microalgae, flagellates, yeasts, rotifers, copepods, plankton, brine shrimp nauplii and fertilized oyster or mussel eggs, has been used to feed shrimps during their protozoeal, mysis and postlarval stages (Kittaka 1975; Liao *et al.* 1983). In addition, shrimp larviculture is made more convenient, but with variable success, through the use of prepared and artificial feeds such as tissue suspensions of fish, crustaceans and molluscs (Tacon 1990; Alikunhi and Ali 1990), and microencapsulated, microparticulate or flaked artificial larval feeds (Jones *et al.* 1979; Kanazawa *et al.* 1982; Bautista *et al.* 1989; New 1990). However, the use of live or prepared feed of bacteria has never been promoted even though they are small, prolific and nutritious (Kobayashi and Kurata 1978; Shipman *et al.* 1975).

Phototrophic bacteria represent such a group of potential candidates for use as feed in aquaculture (Noparatnaraporn et al. 1986; Kobayashi and Tchan 1973). The various species of purple nonsulfur bacteria (Rhodospirillaceae) contain a variety of carotenoids which give cell cultures a distinct coloration of brown, red, red-brown, pink, purple and violet (Imhoff 1992). Their cell membranes contain bacteriochlorophyll which converts light energy into useful

chemical energy (Gest 1993). Besides being widely distributed in nature, phototrophic bacteria play a major role in purifying various organic wastes in the natural environment (Kobayashi and Kondo 1984). They are also important sources of high quality protein and other active substances (Kobayashi and Kurata 1978). Large amounts of cellular vitamin B_{12} and folic acid have been recorded in phototrophic bacteria (Sasaki *et al.* 1991).

The aims of this study are to elucidate the nutritional value of an indigenous strain of phototrophic bacteria, *Rhodopseudomonas palustris* strain B1, and to assess its toxicity and acceptability as an aquaculture feed or supplement. We used *Artemia* or brine shrimp as our test model in the feeding experiments. This is because *Artemia* is the most frequently used and the most convenient form of live food used for aquaculture (Tacon 1990). Besides, *Artemia* culture is simple to manage and the animal grows to maturity in 8 days. Its larvae, which are readily obtainable from commercially produced cysts, are filter feeders of microscopic food particles.

Materials and Methods

Culture of Bacteria

Rhodopseudomonas palustris strain B1 was isolated from starch noodle processing wastewater and maintained in the laboratory. The bacterial cells were mass cultivated in stoppered, 1-l Schott bottles containing the culture medium under anaerobic-light conditions (Getha 1995). The culture medium was made of diluted (50%) sago decanter wastewater enriched with mineral salts and yeast extract (Getha 1995). Anaerobic-light conditions and continuous mixing were provided by tungsten lamps (4 klux) and a rotary shaker (130-140 rpm), respectively.

Bacterial cells were harvested after four days of growth by centrifuging. The cells were then dried in the oven at 60°C for 24 hours. The dried cell mass was grounded in a blender and further pounded to powder in a mortar.

Proximate Analysis

Proximate analysis of the dried bacterial powder was carried out using standard methods (AOAC 1990). In addition, amino-acid analysis was performed using high-performance liquid chromatography (HPLC). Three methods of hydrolyzing the samples were used. Performic acid oxidation was used for the analysis of cysteine and methionine. Alkaline hydrolysis was used solely for the analysis of tryptophan. All the other amino acids were analyzed using the 6N HCl hydrolysis method.

Feeding Tests on Artemia Shrimps

The following four diets were formulated:

1) A combination of particulate feed (PF) for prawn larvae (Higashimaru Foods Inc., Japan) plus *Spirulina* sp. (Sp), a dried blue-green algae (this combination was used as a control as it was the best in preliminary feeding trials);

Table 1. Composition of experimental diets fed to Artemia larvae.

Diet	Diet composition	Feeding density (g/day)	Crude protein* (g CP/day)	Crude fat (g CF/day)
Diet 1	Prawn feed (PF) +	0.10g PF	0.08	0.010
	Spirulina (Sp)	0.06g Sp		
Diet 2	PF + Dried biomass (Db)	0.10g PF 0.08g Db	0.08	0.008
Diet 3	PF + Fresh biomass (Fb)	0.10g PF 0.55g Fb	0.08	0.008
Diet 4	Db	0.20g Db	0.08	0.001

PF - 48% CP, 7.0%CF (Higashimaru Food Inc.);
Sp - 60% CP, 4.8%CF (Ciferri and Tiboni 1985);
Db - 40% CP, 0.64%CF ;
Fb - 85% moisture (Data from present study).
All values in % dry weight

2) PF plus dried bacterial cells (Db);
3) PF plus fresh bacterial cells (Fb); and
4) Db only (Table 1).

An unfed control was also included.

Second-instar *Artemia* larvae which were collected 24 hours after hatching were used in the feeding tests. Feeding was done in 4-l plastic aquaria containing 2 l of synthetic seawater of 25 ppt salinity at 30° ± 2°C. The larval stocking density was approximately 1 larva.ml^{-1} culture water. Tanks were duplicated for each diet, including blanks (no addition of any food). The feeding rate was twice daily giving a total of between 0.16 to 0.20 g (dry feed).day^{-1}.tank^{-1}. The diets when given had the same crude protein content of 0.08 g.day^{-1}. Half the volume of the rearing water was exchanged with fresh seawater daily.

The number of surviving larvae was determined daily by subsampling using a 100-ml beaker. Four subsamplings were done. Ten to 20 larvae were sacrificed on day 0 (start of experiment), 1, 3, 5 and 7 for determination of their total body lengths. The lengths were measured under a compound microscope using a calibrated eye-piece micrometer. Feedings were terminated after 7 days of culture. The life-cycle of *Artemia* shrimps from the larval to the adult stage takes 8 days under optimum conditions (Sorgeloos *et al.* 1986).

Statistical Analysis

Larval mortality, which is the inverse of larval survival, was estimated for each treated *Artemia* population, based on the decaying exponential function (see Pauly 1983) as follows:

$$N_t = N_o\, e^{-zt}$$

where N_o = initial population size,
N_t = population size at time t,
z = instantaneous mortality rate.

Statistical differences among the estimated z values for each diet was then tested using an analysis of covariance (ANCOVA) (Zar 1984).

Differences in growth of body length among diets were statistically tested using analysis of variance and, if found to be significantly different, further subjected to a multiple range test (LSD). The condition of homogeneity of variance was satisfied (p=0.269) by testing the Cochran C statistic using the computer software Statistica™ (Cochran C=0.4334; df=3).

Results and Discussion

Characteristics of Rhodopseudomonas palustris Strain B1

PHYSICAL ATTRIBUTES

The bacteria are gram-negative, with rod- to ovoid-shaped cells. Cellular dimensions vary from 2.0-2.5 μm length to 0.5-0.7 μm width. Cell color is reddish-brown due to a high concentration of cellular carotenoids (Getha, in preparation).

PROXIMATE COMPOSITION

R. palustris strain B1 grown in sago wastewater for four days were found to contain 40% crude protein, 0.64% crude lipid and 2.09% crude fibre on a dry cell weight basis.

In contrast, the proximate composition of the same strain grown in an enriched standard medium (Dow 1982) showed higher levels of crude protein (56.2%) and lipid (2.78%) [Getha 1995]. The standard medium also produced bacterial cells with a higher true protein content of 55% dry weight, compared to 31% true protein for waste-grown bacteria. These differences in proximate content between waste-grown and standard medium-grown bacteria could be explained by the presence of sago debris still present in the effluent. Nevertheless, the proximate composition of the standard medium-grown bacteria reflects the potential yields of strain B1. Crude protein levels were comparable, if not superior, to other conventional larval prawn foods (Table 2).

AMINO ACID PROFILE

The amino acid profile of waste-grown strain B1 is shown in Table 3. The amino acid levels were lower for bacteria grown in wastewater compared to those grown in the standard medium. The dietary essential amino acids (EAA) for penaeid shrimps, namely, lysine, histidine, threonine, valine, methionine, isoleucine, leucine, phenylalanine, arginine and tryptophan (Tacon 1990), of strain B1 comprised 53% of the true protein present, regardless of the culture media used.

Levels of all EAAs present in the waste-grown bacteria were within the dietary requirements of penaeid shrimps at the 35-55% dietary protein level (Table 3).

Table 2. Proximate composition of some larval prawn food

Food species/ type	Percentage dry weight matter			Reference
	Crude protein	Carbohydrate	Crude lipid	
Skeletonema costatum	37.0	21.0	5.0	Parsons et al. (1961)
Thalassiosira weisflogii	44.5	26.1	11.8	Emmerson (1980)
Chaetoceros calcitrans	23.9	19.0	8.7	Tobias-Quinitio & Villegas (1982)
Chaetoceros simplex	22.8	21.8	18.1	Kanazawa (1969)
Isochrysis galbana	27.9	8.8	16.1	Ben-Amotz et al. (1987)
Spirulina sp.	58.6	22.7	4.8	Tacon (1990)
Bakers yeast	39.0	39.1	8.0	Oura (1983)
Marine yeast	34.0	11.1	1.7	Oosawa & Kawano (1971)
Artemia	55.2	18.1	12.5	Tobias-Quinitio & Villegas(1982)
Mytilus edulis	57.2	20.4	4.6	Sedgwick (1979)
Hen egg yolk	31.7	negligible	67.0	Cotterill & Glauert (1979)
Particulate prawn feed (PF)	48.0	na	7.0	Higashimaru Foods Inc., Japan
Rhodopseudomonas palustris*	[1]56.2 [2]40.0	[1]25.0 na	[1]2.78 [2]0.64	Getha (1995) present study

Sources: Chong 1993; *Getha 1995.
[1]Grown in enriched standard media
[2]Grown in sago waste water
na: not analyzed

Survival of Artemia *Shrimps Fed on Strain B1*

Survival of the *Artemia* larval populations fed with either mixed *Spirulina*-prawn feed (Diet 1), mixed dried bacteria-prawn feed (Diet 2), or mixed fresh bacteria-prawn feed (Diet 3) gradually decreased over the first five days, with very little difference among their survival rates (Table 4). The solely bacterial diet (Diet 4) gave a slightly lower survival rate. However, in tanks where the larval populations were not given the diets (blank), the larval population dropped drastically after the second day and almost all brine shrimps died by the fifth day. After the fifth day, survival of fed brine shrimps started to drop substantially regardless of the diet given. After seven days, the highest brine shrimp survival (60%) was recorded for Diet 1, followed by Diet 2 (53%), Diet 3 (44%) and Diet 4 (42%).

The survival of the brine shrimp populations fed on the four diets, including the 'blank', can alternatively be expressed in terms of their daily instantaneous mortality rate, z (Table 5). The negative exponential fit to the survival curves showed very good fits ($p<0.01$). However, ANCOVA indicates that the differences among the mortality rates of the fed brine shrimp populations were not significant ($F=0.85$; $p>0.5$). Therefore, the bacterial cells when given as the sole diet, or given as a supplement (mixed diet), resulted in comparable brine shrimp survivals. The results indicate that *R. palustris* strain B1 was not toxic to the brine shrimps.

Table 3. Amino acid profiles of *Rhodopseudomonas palustris* strain B1 grown in standard medium (I) and sago wastewater (II), and the dietary EAA requirement of penaeid shrimps at 35-55% dietary protein level .

Amino acid (% dry weight)	Strain B1#		Dietary EAA@ (% of dry diet)
	I [1]	II [2]	
Lysine*	2.60	1.88	1.80 - 2.83
Histidine*	1.02	0.70	0.54 - 0.85
hreonine*	2.38	1.47	1.81 - 1.83
Valine*	3.21	2.02	1.04 - 1.64
Methionine*	1.26	0.58	0.66 - 1.04
Isoleucine*	2.21	1.42	0.83 - 1.31
Leucine*	5.16	2.82	1.71 - 2.69
Phenylalanine*	5.99	2.51	0.94 - 1.48
Arginine*	3.93	2.24	1.90 - 2.98
Tryptophan*	1.04	0.71	0.21 - 0.52
Aspartic acid	5.05	3.15	
Serine	2.00	1.24	
Glutamic acid	6.50	3.91	
Proline	2.36	1.30	
Glycine	3.09	1.76	
Alanine	4.37	2.33	
Tyrosine	2.39	1.00	

*Essential amino acids (EAA)
Getha (1995)
@ Tacon (1990)
[1] contained 55% true protein
[2] contained 31% true protein

Growth of *Artemia* Shrimps Fed on Strain B1

The increase in total body length of *Artemia* shrimps fed on the various diets is shown in Table 6. Brine shrimps fed on Diet 1 (without bacteria) achieved an increase in body length of 118% after 7 days of rearing. Brine shrimps fed solely on dried bacteria cells (Diet 4) achieved a length increment of 78% while those on mixed diets of bacteria and particulate prawn feed achieved increments of 80% (Diet 2) and 88% (Diet 3) during the same period. ANOVA performed on the body lengths at day 7 show significant differences ($F=14.0$; $p<0.01$). However, a further multiple range test indicates that brine shrimps fed with bacteria, either solely or as supplement (Diets 2, 3 and 4), had closely similar growth rates which were significantly lower than the growth rate of those fed Diet 1.

Discussion

The preliminary studies indicate that the waste-grown cells of *Rhodopseudomonas palustris* strain B1 have the potential to be used as an aquaculture feed for brine shrimps. The phototrophic bacterial feed is non-toxic to brine shrimp larvae. Survival of larvae fed with bacterial cells given either solely or as a supplement was not significantly different to survival of larvae fed with the control diet of *Spirulina* mixed feed . However, larvae fed with the bacterial diets showed significantly lower growth than larvae fed on the control

Table 4. Effect of different experimental diets on the survival rate of *Artemia* larvae.

Diet*		Sampling day							
		0	1	2	3	4	5	6	7
PF + SP	Survivors#	2035 ±5.7	2105 ±11.3	1955 ±7.9	1875 ±8.3	1825 ±10.0	1730 9.1	1555 ±10.9	1235 ±8.3
	% survival	100%	103%	96%	92%	90%	85%	76%	61%
PF + Db	Survivors	1990 ±4.5	2100 ±12.5	1895 ±9.7	1865 ±5.5	1850 ±9.5	1710 ±10.0	1360 ±9.0	1045 ±4.3
	% survival	100%	106%	95%	94%	93%	86%	68%	53%
PF + Fb	Survivors	1865 ±10.4	1815 ±13.7	1820 ±10.9	1660 ±7.6	1570 ±10.8	1545 ±7.9	1255 ±7.6	815 ±5.4
	% survival	100%	97%	97%	89%	84%	83%	67%	44%
Db	Survivors	2180 ±8.9	2040 ±3.8	1865 ±12.4	1815 ±1.3	1770 ±3.5	1640 ±5.4	1510 ±6.6	920 ±1.8
	% survival	100%	94%	86%	83%	81%	75%	69%	42%
Blank	Survivors	2200 ±9.9	2040 ±7.1	1840 ±2.8	500 ±4.2	70 ±0.7	20 ±0.7	0	0
	% survival	100%	93%	84%	23%	3%	1%	-	-

*PF = Prawn feed; SP = Spirulina; Db = Dried biomass (strain B1); Fb = Fresh biomass (strain B1); Blank = Non-addition of food.
#Mean number of surviving larvae from 4 sample readings.

Table 5. Effect of different diets on daily instantaneous mortality rate (z) of *Artemia* larvae.

Diet	Mortality rate (z)	r^2 (%)	Sum of squares (from ANOVA Table)		F-ratio	Prob. level
			Reg SS	Total SS		
PF + Sp	0.076	77.8	0.324486	0.416958	42.10822	0.00003*
PF + Db	0.100	77.5	0.563905	0.727358	41.39942	0.00003*
PF + Fb	0.114	68.9	0.733500	1.064993	26.55266	0.00024*
Db	0.103	72.7	0.593390	0.815761	32.02165	0.00011*
Blank	1.238	94.1	15.339912	16.296795	48.09340	0.00615*

*Statistically significant difference ($p<0.05$) for null hypothesis, Ho: z (regression coefficient)=O
r^2 measures the strength of the fitted regression of ln N on t.

Table 6. Effect of different experimental diets on the growth of *Artemia* larvae.

Diet*	Mean body length (± s.d) in mm			
	Day 0	Day 3	Day 5	Day7
PF + Sp	0.78±0.06	1.31±0.07	1.53±0.19	1.73±0.18
PF + Db	0.79±0.06	1.30±0.13	1.40±0.10	1.42±0.10
PF + Fb	0.78±0.08	1.29±0.17	1.43±0.12	1.47±0.14
Db	0.78±0.11	1.03±0.08	1.32±0.13	1.39±0.09
Blank	0.80±0.15	0.92±0.11	-	-

*PF = commercial larval prawn feed; Sp = Spirulina; Db = dried bacteria; Fb = fresh bacteria; Blank = no food.

diet. Interestingly, if bacterial cells are used solely, or as a feed supplement, there is no significant effect whether they are given in fresh or dried form. Kobayashi and Kurata (1978) suggested that, in dried form, the phototrophic bacterial cells are as valuable as fish protein, whereas in the fresh form, the cells are highly nutritious and contain heat-labile, hormone-like growth substances. Although the vitamin content of strain B1 has not been analyzed, such studies on other related species, e.g. *Rhodobacter capsulatus* and *Rhodopseudomonas sphaeroides*, have shown high cellular concentrations of vitamin B complex (Sasaki *et al.* 1991) and vitamin E (Noparatnaraporn *et al.* 1986).

The high value protein of strain B1 which contains 53% EAA out of the whole true protein content is comparable to other protein-rich food sources, e.g., *Chlorella* and yeast, which also contain about 53% EAA (Kobayashi and Kurata 1978). Methionine and phenylalanine, which are the limiting EAA in most animal feedstuffs, are present in high concentrations even for waste-grown bacteria cells (0.58% and 2.51%, respectively) [see Table 3]. In comparison, yeast has 0.51% and 2.2%; *Chlorella*, 0.27% and 2.65%; soybean, 0.43% and 1.98%, respectively (Kobayashi and Kurata 1978; Sasaki *et al.* 1991). The non-essential amino acids, cystine and tyrosine, are synthesized from methionine and phenylalanine, respectively (Tacon 1990).

Differences in growth between brine shrimps fed on bacteria (Diet 4) or bacteria-supplemented diet (Diets 2 and 3) and the bacteria-free diet (Diet 1)

could be attributed to differences in proximate composition and fatty acid content of the diets. Commercial diets low in protein and lipid levels are known to cause low survival and growth of penaeid shrimps (Kanazawa *et al.* 1982; Bautista *et al.* 1989). However, the crude protein levels of all four diets were similar, and hence, the observed growth difference is likely due to the difference in lipid level.

The waste-grown strain B1 has a low lipid content of 0.64%, whereas *Spirulina* which was added as a supplement in Diet 1 has a lipid content of 4.8% (Tacon 1990). Lipid and fatty acids are very important energy sources for shrimp development, and are essential components of cellular membranes and hormones (Jones *et al.* 1979; Tacon 1990). Polyunsaturated fatty acids (PUFA) such as linoleic (18:2n-6) and gamma linolenic (18:3n-3) fatty acids are necessary for shrimp growth. However, supplementing the highly unsaturated fatty acids (HUFA), eicosapentaenoic acid or EPA (20:5n-3), and docosahexaenoic acid or DHA (22:6n-3), to the diet produces optimal growth and survival (Kompiang 1990). This is because HUFA are responsible for key metabolic functions (Tacon 1990) and shrimps have limited ability to synthesize them *de novo* from neutral lipids (Clarke and Wickins 1980; Read 1981). Dietary phospholipids also exert a beneficial effect on the growth and survival of shrimps, especially in conjunction with the availability of PUFA and HUFA (Tacon 1990).

It appears that low fat content in waste-grown bacterial biomass could be a limiting factor in shrimp culture. However, bacterial biomass would be a good supplement to diets rich in fat but poor in protein content. This study elucidates on the food value of *R. palustris* strain B1 and its acceptability to *Artemia* larvae as a feed. It also provides the basis for future studies, e.g., to determine the optimum concentration of bacterial biomass in the feed formulation and to assess the suitability of the bacterial biomass in the culture of penaeid shrimps and fishes. Given the need for more protein food, and growing concerns over the role of agricultural wastes in water pollution in Asian countries, the utilization of phototrophic bacteria cultured in wastewaters may yet prove to be an answer to our woes.

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