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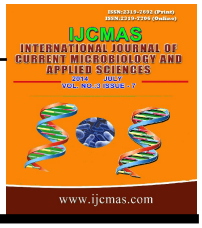

Original Research Article

Studies on the isolation and characterisation of marine yeast, β –glucan production and immunostimulatory activity on *Carassius auratus*

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A B S T R A C T

The present investigation was aimed to study about the marine yeast isolates and their growth, β- glucan production and microbial load of juvenile goldfish. Five different yeast species were isolated based on the morphological characteristics. From the Vizhinjam coast in Southern Kerala of India, three different species could be found and two from the Rajakkamangalam coast in Kanyakumari district of Tamilnadu, India. Generic level identification of isolated marine yeasts was carried out through microscopic examination, lactophenol cotton blue staining and selected biochemical tests. The marine yeast isolates were identified specifically by their growth on corn meal agar for chlamydospore production or true hyphae production and they are identified as *Trichosporon* species, *Candida parapsilosis*, *Cryptococcus* sp., *Torulopsis glabrata* and *Candida albicans*. The production of protein, free amino acids and carbohydrates were also screened among the different yeast species for evaluating the balanced nutrient content. The immunostimulatory activity of each marine yeast species can be determined by the experiment on gold fish *Carassius auratus* and it is clear that the total plate count (TPC) was much higher in the gold fish *Carassius auratus* soon after the development of pathological symptoms of specific fish pathogen administered. Interestingly, the fish inoculated with marine yeast sp (1–5 treatments) showed a reduction in bacterial load i.e., the total *pseudomonal* count

Keywords

β- glucan,
Juvenile goldfish,
Trichosporon sp,
Candida parapsilosis,
Cryptococcus sp,
Torulopsis glabrata,
Candida albicans,
Total plate count (TPC).

Introduction

Yeast is an eukaryotic microorganism classified in the kingdom fungi; with about 1,500 species so far described dominate fungal diversity in the oceans. Yeasts are a polyphyletic group of basidiomycetous and ascomycetous fungi with a unique characteristic of unicellular growth. The term "yeast" is derived from the Old Dutch word gist and the German word gischt, which refers to fermentation (Kurtzman and Fell, 1989). Yeasts are ubiquitous in their distribution and populations mainly depend on the type and concentration of organic materials. There are approximately 100

genera and 800 described species of yeasts and estimates suggest that these numbers represent only about 1% of the species that exist in nature, the rest being non-culturable (Fell, 2001).

Obligate Marine yeasts are those yeasts that, thus far, have never been collected from anywhere other than the marine environment, whereas 'facultative marine' yeasts are also known from terrestrial habitats. The discovery of marine yeasts goes back to 1894, when Fisher separated red and white yeasts from the Atlantic Ocean and identified them as *Torula* sp. and *Mycoderma* sp., respectively. Following Fisher's discovery, many other workers, such as Bhat and Kachwala, (1995); Van Uden and Zobell, (1962); Van Uden and Fell, (1968) isolated marine yeasts from different sources, viz., seawater, marine deposits, seaweeds, fish, marine mammals and sea birds (Flannigan, 1974). The most important genera of marine yeasts include *Candida* spp., *Cryptococcus* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Metchnikowia* spp., *Kluyveromyces* spp., *Rhodosporidium* spp., *Pichia* spp., *Hansenula* spp., *Saccharomyces* spp., *Trichosporon* spp. and *Torulopsis* spp (Munn, 2004).

Marine yeast are distributed in almost every part of the aquatic environment i.e., oceans and seas, estuaries, lakes and rivers. Truly marine yeast must be able to grow on or in a marine substrate. Sarlin (2005) observed yeast on most of their culture plates inoculated with samples of marine materials collected from land as well as from the open ocean. The morphological characteristics of yeast in general are subject to the limitations of variation which are not infrequent in culture. Physiological characteristics as a rule are less variable and have been extensively used in yeast systematics (Scheda and Yarrow, 1996). Physiological factors used for classifying yeasts are chiefly the ability to:

- Ferment sugars anaerobically;
- Grow aerobically with various compounds, such as a sole source of carbon or nitrogen;
- Grow without an exogenous supply of vitamins;
- Grow in the presence of NaCl or glucose;
- Grow at 37°C;
- Grow in the presence of cycloheximide;
- Split fat;
- Produce starch-like substances;
- Hydrolyze urea;
- Form citric acid.

In the production of β- glucans, (1-3) - - Glucans are widely distributed in nature, especially in yeast, fungi and algae, which serve a variety of biological functions. They form the major structural components of cell walls, they act as storage carbohydrates and they sometimes play a protective role. This glucan is composed of a (1-3) - β -linked D-glucan backbone to which single D-glucosyl residues are attached β - (1-6) to every third main-chain residue. It has been claimed that yeast β- glucan substantially enhances the function of the immune system by activating macrophages, one of the primary defenses of the immune system (Litchfield and Floodgate, 1975).

Trade in freshwater and marine live ornamental species represents an important market and supply source for the domestic aquatic ornamental industry. The global trade in the ornamental fish is estimated at Rs. 5000 crores, of which India has a minuscule Rs. 2.0 crore. Ornamental fish kept in aquariums are susceptible to numerous diseases (Ahilan *et al*., 2004).

Due to their generally small size and the high cost of replacing diseased or dead fish, the cost of testing and treating diseases is often seen as more trouble than the value of the fish. Due to the artificially limited volume of water and high concentration of fish in most aquarium tanks, communicable diseases often affect most or all fish in a tank. Diseases can have a variety of causes, including bacterial infections from an external source such as *Pseudomonas fluorescens* (causing Fin rot and Fish dropsy)

Immunostimulants can be defined as substances that improve a fish's protection from diseases by stimulating its non-specific immune responses. They react on first contact with a pathogen to slow or stop the establishment of an infection. Immunostimulants are derived from many sources yeast (glucans), other fungi (glucans and chitin), invertbrates (chitin and chitosan), bacteria (LPS) mammals (lactoferrin and transferrin) or they may be artificial (levamisole). The vast majority of immunostimulants' studies with fish have shown major benefits, either by elevating the non-specific defense mechanisms of fish prior to exposure to a pathogen or improved survival following exposure to a specific pathogen when treated with them (Ballou,1974).

Materials and Methods

Collection of samples

Water samples were collected in sterile polythene bags in the morning from the study sites such as Vizhinjam and Rajakkamangalam coast in the South- West coast of India. All the samples were brought to the laboratory in iced-chest. Microbiological analyses were made within 4–6 hrs of sampling.

Media preparation

For the isolation of marine yeast YPDA (yeast peptone dextrose agar) medium (Hi media, Mumbai) was used containing 2.0% and 1.0% yeast extract with 1.5% NaCl. The media were sterilized at 121°C for 15 minutes by autoclaving.

Isolation of marine yeast

One milliliter of water was serially diluted in aged sea water and further plated with YPD agar respectively. After solidification, the plates were incubated in an inverted position at room temperature for 24 hrs for the enumeration of marine yeasts. After attaining visible growth, the yeast colonies appeared on the media was counted. All the determinations were carried out in triplicates and the results are expressed as counts per gram dry weight of soil or per milliliter of water samples. Different morphological colonies were picked up from the petriplates and restreaked thrice in an appropriate agar plates before a pure culture was established in agar slants.

Culture maintenance

Isolated cultures were maintained in respective agar slants at 4°C for short periods (3–6 months). For long periods, agar slants are stored in liquid nitrogen at −60 to −80°C under low oxygen tension or in 5% DMSO (w/v) or as glycerol stocks.

Identification of isolated marine yeasts

Generic identification of isolated marine yeasts were carried out through morphological characteristics, microscopic examination, lactophenol cotton blue staining and selected biochemical tests (Botes *et al.*, 2007).

Growth on corn meal agar for chlamydospore or true hyphae production

The marine yeast isolates were identified specifically by their growth on corn meal agar for chlamydospore production or true hyphae production.

a) 1.9 grams of corn meal agar (Hi media) was prepared and suspended in 100 ml distilled water and boiled to dissolve the agar. To that, one ml tween 80 was added, mixed well and sterilized by autoclaving at 121°C for 15 minutes and pour plate and allowed to solidify.

b) From the marine yeast isolates, a small portion of a single colony of the yeast was picked and inoculated into the corn meal agar.

c) Make few cuts into the agar with the loop in a slanting manner near the secondary and tertiary streak area and place a cover slip over the cut area and the plates were incubated at 30°C for 24–72 hours.

d) The plates were examined under the microscope (100X, 450X) for the characteristic blastoconidia (yeast cells) formation along the streaked line and observed the presence of pseudohyphae, true hyphae and chlamydospores.

Growth optimization of marine yeasts

In order to optimize the yeast growth, the yeast cultures were inoculated in the YPD broth maintained at different pH ranging from pH 6.0, pH 7.0 and pH 8.0. These media were then sterilized, cooled and inoculated with five different yeast cultures and the growth was determined by measuring the optical density for 6 days. The growth of all the cultures was determined by taking the optical density in a spectrophotometer at 510 nm.

Optimization of β- glucan production in different marine yeasts

The production of β- glucan was optimized at different parameters like pH for maximum production. In order to optimize the β-glucan production, the yeast cultures were inoculated in the YPD broth maintained at different pH ranging from pH 6.0, pH 7.0 and pH 8.0 using 1N HCl and 1N NaOH. The temperature can be optimized by growing on different temperatures such as 20°C, 30°C and 40°C. These media were then sterilized, cooled and inoculated with five different yeast cultures respectively followed by incubation.

Production of β- glucan

a) The milled sample (approximately 100 mg, weighed accurately) was passed through a 0.5 mm screen to a 20×125 mm culture tube.

b) To each tube, 1.5 ml of concentrated hydrochloric acid (37% v/v) was added and stirred vigorously on a vortex mixer and the tubes were kept in a water bath at 30°C for 45 minutes and stir them on a vortex mixer.

c) The contents of each tube were transferred to a 100 ml volumetric flask using 200 mM sodium acetate buffer (pH 5.0) to adjust the volume and mixed thoroughly by inversion.

d) The aliquot of each suspension was filtered through a Whatman GF/A glass fibre filter paper and 0.1 ml of

a mixture of exo-1,3- β - glucanase (20 U/ml) and β - glucosidase (4 U/ml) in 200 mM sodium acetate buffer (pH 5.0) was added to the bottom of each tube, contents mixed and incubated at 40°C for 60 minutes.

e) This was followed by the addition of 3.0 ml of glucose oxidase/peroxidase mixture (GOPOD) to each tube and incubated at 40°C for 20 minutes and the absorbance of all solutions were read at 510 nm against the reagent blank.

Production of protein and amino acids in marine yeast sp

The protein content of the isolated marine yeasts were estimated using Folin-Ciocalteau method and the amount of protein present in the samples and control were found out by incorporating the values in a protein standard graph. The aminoacid content of the isolated marine yeasts were estimated by Ninhydrin method from the intensity of colour developed and was read against the reagent blank at 570 nm using a spectrophotometer.

Immunostimulatory effect on ornamental fishes (*Carassius auratus*)

a) Healthy goldfish weighing from 0.257 to 0.326 g taken from the acclimatization tank was stocked in experimental glass tubs of 3 liters of water capacity. The fishes were stocked in all the tubs (in triplicates) at the rate of 2 per tub. The cultivated marine yeast isolates were filtered using a crude filter paper and powdered and mixed thoroughly using a mixer grinder and passed through 250 µ sieve for uniformity.

b) All the ingredients were mixed well with water and autoclaved for 30 minutes. Each marine yeast paste was inoculated into the respective culture tub.

c) The feed was made into powdered form and fed to the juvenile fish at the rate of 10% of their body weight.

d) This was followed by the addition of 24 hr old virulent pathogenic cultures of *Pseudomonas fluorescence* at the rate of one ml per tub. Physico-chemical parameters such as temperature, pH were estimated following standard procedures.

e) The fishes were dissected to remove the intestine, after washing the ventral surface area of the fish with sterile distilled water and the bacteriological analysis of the gut, tissue and fin of the fishes were enumerated.

f) The entire fish parts were homogenized in 9 ml of the diluents (Phosphate buffer saline, 0.01 M) and mixed with a vortex mixer and used as 10^{-1} dilution. Each sample was serially diluted, using pour plate method, poured into four different media.

g) The total plate count (TPC) was determined using Nutrient Agar (NA) media. The total pseudomonad count was determined by replica plating on cetrimide agar (Hi Media, Mumbai) and the inoculated plates were incubated at 37°C and 28 ± 2°C for 3 days.

Results and Discussion

The water samples were collected from the study as vizhinjam and rajakkamangalam coast in the South- west of India and subjected to isolation and enumeration of marine yeast sp. They are presented in the Table 1. From the table, it is clear that the Rajakkamangalam coast (9.6x104) shown the comparatively higher population of marine species than the Vizhinjam coast (6.6x104). Morphological characterizations of marine yeasts based on the colony morphological characteristics are given in the Table 2. However, from the Vizhinjam coast, three different species could be found and two from the Rajakkamangalam coast.

Generic level identification of isolated marine yeasts was carried out through microscopic examination, lactophenol cotton blue staining and selected biochemical tests. The marine yeast isolates were identified specifically by their growth on corn meal agar for chlamydospore production or true hyphae production and the results are given in the Table 3.

From the Table 4, it is clear that the growth of marine species varies it the species. The species 1, species 2 and species 5 gave maximum growth at pH 7, where as the maximum growth for species 3 varied with pH. The species 4 (*Torulopsis glabrata*) showed maximum growth at pH 6 (Table 4).

The production of β- glucan was optimized at different parameters like pH for maximum production on their maximum growth period and the results given in Table 5. The species 1 (*Trichosporon* sp) gave maximum production on pH 6.0. The production of β-glucan was found maximum for both species 2 and 3 at the pH 6 for species 4 (*Torulopsis glabrata*) and 5 (*Candida albicans*).

The production of protein, free amino acids and carbohydrates were also screened among the different yeast species for evaluating the balanced nutrient content. The protein content was high in the marine yeast species 3 (*Cryptococcus* sp) followed by species 5 (*Candida albicans*) at the rate of 58 and 54 mg/I respectively. The free amino acid production was maximum in species 2 (*Candida parapsilosis*) at the rate of 108 mg/ml; whereas the production of carbohydrate was maximum in species 3 (*Cryptococcus* sp) at the rate of 54 mg/ml.

The immunostimulatory activity of each marine yeast species can be determined by the experiment on gold fish *Carassius auratus* and which was serially diluted, using pour plate method, after 21 days of experiment. The total plate count (TPC) was determined and the results are given in Table 7. In the control value, it is clear that the TPC was much higher in the gold fish *Carassius auratus* soon after the development of pathological symptoms of specific fish pathogen (fin rot can be the result of a bacterial infection (*Pseudomonas fluorescens*, which causes a ragged rotting of the fin).interestingly, the fish inoculated with marine yeast species (1–5 treatment) showed a reduction in bacterial load.

Yeast is a polyphyletic group of basidiomycetous and ascomycetous fungi with a unique characteristic of unicellular growth. Yeast is rich with proteins, lipids and vitamins. Yeasts also have immunostimulatory properties by virtue of their complex carbohydrates and nucleic acid components. They can be produced very efficiently and economically because of their shorter generation time and use of inexpensive culture media (D'Souza, 1972).

Yeasts are distributed in almost every part of the aquatic environment (Fell, 2001). Truly

marine yeast must be able to grow on or in a marine substrate. Salinity tolerance does not distinguish marine species from terrestrial species because almost all yeast can grow in sodium chloride concentrations exceeding those normally present in the sea. In this study, the marine yeast species were isolated using YPD Agar supplemented with NaCl. From the results, it was found that yeast species are abundantly seen in the coast of Vizhinjam and Rajakkamangalam. From the table, it is clear that the Rajakkamangalam coast shown the comparatively higher population of marine species than the Vizhinjam coast. This may be due to the congenial environmental factors existing in the region (Table 1).

Physiological factors used for classifying yeasts are chiefly the ability to ferment sugars anaerobically, grow aerobically with various compounds, such as a sole source of carbon or nitrogen, grow without an exogenous supply of vitamins, grow in the presence of NaCl or glucose, grow at 37°C, grow in the presence of cycloheximide, split fat, produce starch like substances, hydrolyze urea, and form citric acid.

In this study, generic identification of isolated marine yeasts were carried out through morphological characteristics, microscopic examination, lacto phenol cotton blue staining and selected biochemical tests by following the standard methods (Norkrans, 1966). Baenett *et al.*, (1990) studied the on the characterization of marine yeast. The growth of yeasts in various assimilation media was examined in both standing and shake culture.

In order to optimize the yeast growth, the yeast cultures were inoculated with in the YPD broth maintained at different pH ranging from pH 6.0, pH 7.0 and pH 8.0. The yeast culture medium was prepared and the pH was maintained in the medium using 1N HCl and 1N NaOH. As expected in the study, it was observed that the species 1 (*Trichosporon* sp) showed maximum growth at 72 hrs of growth at room temperature followed by species 5 (Table 4). From the Table 5, it is clear that the growth of marine species varies with the species. The species 1 (*Trichosporon* sp) species 2 (*Candida parapsilosis*) and species 5 (*Candida albicans*) gave maximum growth at pH 7. The species 4 (*Torulopsis glabrata*) showed maximum growth at pH 6 (Table 5).

1,3- β -Glucans are widely distributed in nature, especially in yeast, fungi and algae, which serve a variety of biological functions. They form the major structural components of cell walls, they act as storage carbohydrates and they sometimes play a protective role by forming at specific sites in response to particular high molecular weight β- glucan, produced by the fungus, *Botrytis cinerea* (grey roy). This glucan is composed of a (1-3) - β - linked D-Glucan backbone to which single D-glucosyl residues are attached β - (1-6) to every third main-chain residue. The production of β- glucan was found maximum in pH 6 and 7 by species 2 and 3. Likewise, the production of β- glucan was high at pH 6 for species 4 and 5 (Table 5).

Studies of (Kirk and Gordan, 1988) revealed the effect of 1,3 and 1,6- β- D-gluco oligo- and poly -saccharides with different structures on the developing embryos of sea urchin, with a molecular mass of between 6–10 kDa and at concentrations. The study of Barnett (1968) supports the findings that the marine yeasts are rich in β- glucans. They reported the effect of 1,3 and 1,6- β- D-gluco oligo- and poly- saccharides with different structures on the developing embryos of sea urchin, *Strongylocentrotus intermedius*. The production of protein, free

amino acids and carbohydrates were also screened among the different yeast species for evaluating the balanced nutrient content. They play a vital role in the immunostimulatory activity of the marine species.

Ornamental fish kept in aquariums are susceptible to numerous diseases.

Table.1 Enumeration of marine yeasts from the sampling sites

Sampling site	Dilution	Colony counts (P1)	Colonycounts (P2)	TVC (CFU/ml)
Vizhinjam (Lat. 8^0 22' N Long. 76^0 57' E)	10^{-1}	TNTC	TNTC	6.6×10^4
	10^{-2}	TNTC	TNTC	
	10^{-3}	81	50	
	10^{-4}	TLTC	TLTC	
Rajakkamangalam (Lat. 8^0 04' N; Long. 77^0 32' E)	10^{-1}	TNTC	TNTC	9.6×10^4
	10^{-2}	TNTC	TNTC	
	10^{-3}	108	84	
	10^{-4}	TLTC	TLTC	

Table.2 Morphological characterization of marine yeasts from the sampling sites

Sampling site	Culture no.	Colony morphology	Total no. sp.
Vizhinjam	1	Small, white, watery colony, circular, entire, glossy, elevated, non-pigmented	3
	2	Moderate, colourless, glossy, white, umbonate,entire margin	
	3	Large, white, watery colony	
Rajakkamangalam	4	Pin headed, white, smooth colony, flat colonies, pale yellow coloured, irregular	2
	5	Moderately large, white, circular, serrated, non-pigmented, flat	

The study of fish diseases has remained a rudimentary branch of veterinary medicine (Ross and Morris, 1965). In some cases the causes of an infection or disease will be obvious (such as fin rot), where, the use of live feed can be used to control the diseases. Morri (1973) demonstrated that the immunity of the fishes can be improved by giving microbes as the live organism. An improper nitrogen cycle, inappropriate aquarium plants and potentially harmful fresh water invertebrates can directly harm or add to the stresses on ornamental fish in a tank (Novozhilova, 1955). Ahilan *et al.*, (2004) found that the influence of probiotics on the growth and gut microbial load of juvenile gold fish. Marine yeast immunostimulants are those substances

obtained from non-host structural materials. They can be considered to be dietary supplements rather than true therapeutic and hence, they have the potential to be widely adopted in animal diets.

Marine yeast is those that can survive longer in sea water than in fresh water. The present study was an attempt to investigate marine yeast isolates and their growth, β-glucan

Table.3 Characterization of marine yeast isolates

Characteristics	**Sp. 1**	**Sp. 2**	**Sp.3**	**Sp.4**	**Sp.5**
Capsule formation	-	-	+	-	-
Urease test	+	-	+	-	-
Pseudohyphae	+	+	+	-	+
True hyphae	+	-	-	-	-
Arthroconidia	+	-	-	±	-
Species	*Trichosporon sp.*	*Candida parapsilosis*	*Cryptococcus sp*	*Torulopsis glabrata*	*Candida albicans*

+ Presence; - absence; ± variable

Table.4 Growth optimization of marine yeast isolates in different pH

Days	***Trichosporon sp.***			***Candida parapsilosis***			***Cryptococcus sp***			***Torulopsis glabrata***			***Candida albicans***		
	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8
1	1.65	3.25	1.67	0.73	0.95	0.79	1.68	1.11	1.02	1.11	1.3	0.95	0.58	0.77	0.54
2	1.82	3.74	1.90	0.92	1.15	1.0	1.74	1.83	1.3	1.53	1.42	1.02	0.75	0.85	0.61
3	2.73	4.56	3.00	2.89	3.22	3.0	3.33	4.2	3.75	2.5	3.17	2.85	1.5	1.98	1.23
4	5.65	6.79	5.87	5.31	6.05	5.83	5.65	5.97	5.5	3.74	4.48	4.03	2.8	3.0	2.65
5	5.75	6.85	5.90	5.40	6.09	5.87	5.7	6.03	5.4	4.00	4.91	4.61	3.66	4.88	3.47
6	3.42	4.72	3.42	4.3	4.77	4.11	4.17	4.66	3.84	3.02	4.0	3.14	3.53	4.67	3.42

Table.5 Optimization of production of β-Glucan in different species of marine yeasts

Marine yeast	**Optical Density** (510 nm)		
	pH 6	pH 7	pH 8
Trichosporon sp.	0.02	0.01	0.01
Candida parapsilosis	0.03	0.03	0.02
Cryptococcus sp	0.04	0.04	0.03
Torulopsis glabrata	0.03	0.02	0.02
Candida albicans	0.02	0.01	0.01

Table.6 Macromolecular contents of marine yeast species

Contents	**Macromolecular contents in marine yeast sp. (mg/ml)**				
	***Trichosporon* sp**	***Candida parapsilosis***	***Cryptococcus* sp**	***Torulopsis glabrata***	***Candida albicans***
Protein	35	23	58	23	54
Amino acids	108	78	85	30	25
carbohydrates	28	26	54	18	7

Table.7 Total plate counts (TPC) of *Carassius auratus*

Treatment	**Physico-chemical factors**		**Total plate count (CFU/g)**		
	pH	**Temp (^{o}C)**	**Tissue**	**Gut**	**Fin**
T1 (Sp.1)	6.5	28.0	$179X\ 10^{4}$	$40X10^{5}$	TNTC
T2 (Sp.2)	6.5	28.0	TNTC	$31X10^{5}$	$112X10^{4}$
T3 (Sp.3)	6.5	28.0	$80X10^{5}$	$62X10^{6}$	TNTC
T4 (Sp.4)	6.5	28.0	TNTC	$92X10^{6}$	TNTC
T5 (Sp.5)	6.5	28.0	$292X10^{4}$	TNTC	$280X10^{4}$
Control	6.5	28.0	TNTC	TNTC	TNTC

production and microbial load of juvenile gold fish. Presently the marine yeast species were enumerated from Vizhinjam and

Rajakkamangalam coasts and identified as *Trichosporon sp., Candida albicans, Cryptococcus sp, Torulopsis glabrata,*and *Candida albicans.* Their growth conditions were optimized in different pH conditions. The β- glucan production was also screened and their active role in the iimmunostimulatory activity in gold fish *Carassius auratus* was undeniably determined. In this study investigation it was found that by the use of marine yeast species, the immunity of the fishes can be improved.

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