Probiotic yeasts with phytase activity identified from the gastrointestinal tract of sea cucumbers

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Abstract

Yeasts are microorganisms commonly found in the gastrointestinal track of animals, and several yeast strains produce phytase. The present study focused on the isolation of yeast species from sea cucumbers and on the yeasts' ability to produce phytase. Two strains of phytase were isolated and identified: *Yarrowia lipolitica* and *Candida tropicalis*. These strains produced high amounts of extra cellular and cell bound phytases. These could be used as probiotic yeasts by the sea cucumber farming industry.

Introduction

The intestines of animals contain large amounts of microorganisms that have specific functions such as catabolic breakdown of fibres and complex nutrients, as well as the production of vitamins. Raibaud (1992) reported that intestinal microorganisms play a role against pathogenic microbes and that the imbalance of gut microorganisms can lead to the rapid growth of opportunistic pathogens that can be harmful to the host animal. The possibility of using intestinal microbiota as probiotics has been reported by Hirimuthugoda et al. (2006). This study was carried out to better understand intestinal microorganisms in sea cucumbers and to develop probiotic microorganisms for sea cucumber farming.

As a main component of DNA, phosphorous is a vital element. Cereals, legumes, fodder and root crops store phosphorous as phytate and phytin, which can only be digested by ruminants; with other animals, undigested phosphorous is released into the environment. Accumulation of undigested phosphorous in soils and waters is toxic.

Phytases can have a significant role in controlling phosphate pollution due to their capability of catalysing the release of phosphate from both phytate and phytin. Recently, microbial plants and animal-derived phytases have been made available as feed supplements. They have become the most popular and widely used enzymes in animal farming systems. However, scientists have not yet studied marine microbial phytases. Therefore, we made an attempt to isolate microbial species from the gut of sea cucumbers to study their ability to secrete phytase.

Materials and methods

Sampling and yeast isolation

Sea cucumbers from coastal areas of Sri Lanka and China were collected randomly and dissected under aseptic conditions. Guts were separated and homogenized, and 2 mL of homogenized samples were placed into 20 mL of liquid YPD (2% glucose, 2% polypeptone, 1% yeast extract and seawater) culture medium, which was treated with antibiotics and cultured at 28°C for five days. After five days, cell cultures were plated on YPD agar plates and yeast colonies were transferred to slants.

Assay of phytase activity

Yeast strains were inoculated into 250 mL flasks with a 50 mL medium containing 0.5% sodium phytate, 1% ammonium sulfate, vitamins and mineral salts, and grown for five days at 28°C. The supernatants were assayed by measuring the amount of phosphate released (Fiske and Subbarow 1925) using sodium phytase as the substrate (Vohara and Satyanarayana 2001). One unit of phytase is defined as the amount of enzyme that liberates 1 mU inorganic phosphate per minute at ambient temperature. The effect of temperature and pH on phytase activity was studied by incubating the enzyme at pH 4–9 (buffers used were 0.2 M Acetate for 4–6 and 0.2% M $Na_2B_4O_7$.10 H_2O-HBr_3 for 7–9) and at temperatures of 37°C, 45°C, 50°C, 55°C, 60°C, 65°C and 70°C.

DNA extraction, PCR and phylogenetic analysis

The total genomic DNA of the yeast strains was isolated and purified by using the method described

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by Sambrook et al. (1989). The 18S rDNA fragment and ITS fragment inserted on the vector were sequenced by Shanghai Sangon Company. The sequences obtained above were aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST). The routine identification of the yeasts was performed by using the method described by Kurtzman and Fell (1998).

Results and discussion

Two yeast strains were isolated and both strains were obtained from the gut of *Holothuria scabra*. Generally, animals have a large number of gut-colonized microorganisms in their gastrointestinal tract. Our results show that the microorganisms were isolated from the gut content and therefore, to our knowledge, these strains cannot be categorized as gut-colonized yeasts. Further research work is necessary on this aspect. Strains were labelled as W2B (from China) and YF12C (from Sri Lanka). Based on the biochemical characteristics and on similar information for the type of strains listed by Kurtzman and Fell (1998), we found that strain W2B and YF12C were similar to *Yarrowia lipolitica* and *Candida tropicalis*. DNA sequences analysis of phylogeny with those in the National Center for Biotechnology Information (NCBI) database further confirmed that the yeast strains obtained in this study were closely related to *Yarrowia lipolitica* (W2B) and *Candida tropicalis* (YF12C). 18S rDNA sequences of yeast strains were deposited in NCBI under the Accession Nos. of DQ 438177 –W2B and DQ 515959- YF12C.

Table 1. Phytase activity of two yeast strains.

Strain	phytase activity (mU min^{-1})	optimum temperature (°C)	optimum pH
W2B	61 ± 0.011^a	60	8
YF12C	49 ± 0.008^a	55	8
	28 ± 0.045^b	65	6

[a] extra cellular phytase

[b] cellbound phytase

It is interesting to mention that phytase-secreting marine yeasts are present in sea cucumber intestines. A few yeast strains that exhibit phytase secretion have been observed (Pandy et al. 2001), but this was the first report of sea cucumber-derived yeast phytases. Strain W2B was able to produce only extra cellular phytase while YF12C was able to produce both extra cellular and cell bound phytases. Vohara and Satyanarayana (2004) studied cell bound phytase from the yeast *Pichia anomala*, which can be used in the animal feed industry to reduce phosphate pollution.

Temperature and pH are the most influential factors on enzyme production in all studies. In this study, high phytase activity was observed at between 55°C and 65°C. For the strain W2B, 60°C was the optimum temperature. For the strain YF12C, the optimum temperature was 55°C for extra cellular and 65°C for cell bound enzyme production. A pH of 8 was the optimum for extra cellular enzyme production for both strains, while pH 6 was the optimum for cell bound enzyme synthesis of YF12C. In general, the optimum pH and temperature are around 4.5–6 and 45–60°C, respectively (Pandy et al. 2001). However, in this study we observed higher pH values, probably because these strains came from the marine environment.

Yarrowia lipolitica has several physiological properties of industrial significance. The species is abundant in the marine environment, and is well known for the production of proteases, lipase and utilization of *n*-paraffin (Kurtzman and Fell 1998). Although much research has been conducted on *Yarrowia lipolitica*, this paper reports on its phytases. This species can be used at the commercial level for marine phytase production. *Candida tropicalis* is a well-known yeast species found all over the world, and is a common pathogenic strain on humans. Therefore, industrial application of this species is limited, although extracted phytase can be used as an industrial product. Present market trends have very clearly indicated that there is a significant demand for phytase as a feed supplement, and various products under different trade names are available. For example, Cenzyme, Natu-phos, and Gist-Brocades are the leading products (Pandy et al. 2001).

The role or impact of yeasts in the gastrointestinal tract of sea cucumbers is not clearly known but obviously the significant phytase synthesis is favourable for digesting phytate phosphorous as well as a probiotic form. In recent decades, sea cucumber farming increased remarkably and phytate-rich feed components are used. Therefore, this phytase synthesing yeast plays a major role in the food digestion of sea cucumbers. Excretion of undigested phosphorous, leads to eutrophication and causes a decrease in water quality at sea cucumber farms. This situation is favourable for pathogenic microorganisms and, therefore, the yeast phytase observed from sea cucumbers has a significant value in the sea cucumber industry, and all phytases found thus far are not from marine sources. The authors of this paper are conducting further research on the application of these two yeast strains in sea cucumber farming and the purification of enzymes in optimized medium.

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An observation on the effect of environmental conditions on induced fission of the Mediterranean sand sea cucumber, *Holothuria arenicola* (Semper, 1868) in Egypt

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Introduction

Holothuria arenicola is the most important and abundant sea cucumber species in the Mediterranean Sea on the Egyptian coast (Fig. 1A). It was recorded in 1984 for the first time on the Egyptian Mediterranean coast (Shoukr et al. 1984). Its habitat extends from the Indo-Pacific to the tropical Western Atlantic. Its reaches a size of about 26 cm. Presently, *H. arenicola* is overexploited in Egyptian waters due to the increasing demand from Asian markets. The loss of sea cucumber stocks is likely to have a significant negative impact on the ecosystem and the adjacent marine environment as a whole. Therefore, there is an urgent need for intensive studies of the biology, culture and fishery management of *Holothuria arenicola*.

Some holothurians are known for their ability to reproduce asexually by fission. Most holothurian species with asexual reproduction follow the twisting and stretching mode (Uthicke 2001). The first trial to induce asexual reproduction in *H. arenicola* was done by Kilada et al. (2000), who investigated the induction of asexual reproduction by using rubber bands. The present work aims to describe the stages of asexual reproduction by fission and the effect of environmental factors on dividing and survival rates.

Method

Asexual reproduction of *H. arenicola* was induced by fitting rubber bands just in front (the upper 45%) of the middle portion of the body (Fig. 1B). Specimens were kept in a tank with a thin layer of fine sand on the bottom. Water salinity was 36 ppt. The tank's water was changed daily, and the number of divided, undivided and dead animals were reported daily.

Discussion and conclusion

Observations showed that the body was more constricted at the constriction point. The posterior part was swollen and extended. The posterior and anterior parts rotated in opposite directions resulting in more constriction until both parts stretched (Fig. 1C) and finally split, although they were still connected to each other via the gut. After one day, the anterior and posterior parts were completely separated (Figs. 1D and 1E). The survival rate of the posterior part was higher than that of the anterior part. The entire process of fission lasted from one to five days.

Because of electrical problems that affected the water aeration, low survival rates were obtained.

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