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Extracellular enzyme production and phylogenetic distribution of yeasts in wastewater treatment systems

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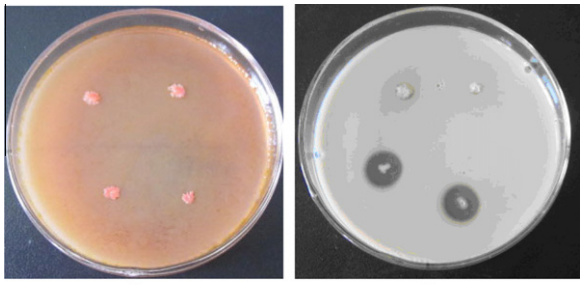
HIGHLIGHTS

- We isolated 257 yeasts from five different wastewater treatment systems.
- The abilities of the yeasts to produce extracellular enzymes were detected.
- The yeasts mainly produced lipase, protease, MnP and LiP.
- The enzyme producing yeasts had diverse phylogenetic distribution.
- The types of enzymes produced by yeasts were related with isolation sources.

GRAPHICAL ABSTRACT



A B

Colony characteristics of yeast isolates that could produce extracellular enzymes (A, producing lipase; B, producing protease)

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ABSTRACT

The abilities of yeasts to produce different extracellular enzymes and their distribution characteristics were studied in municipal, inosine fermentation, papermaking, antibiotic fermentation, and printing and dyeing wastewater treatment systems. The results indicated that of the 257 yeasts, 16, 14, 55, and 11 produced lipase, protease, manganese dependant peroxidase (MnP), and lignin peroxidase (LiP), respectively. They were distributed in 12 identified and four unidentified genera, in which *Candida rugosa* (AA-M17) and an unidentified Saccharomycetales (AA-Y5), *Pseudozyma* sp. (PH-M15), *Candida* sp. (MO-Y11), and *Trichosporon montevideense* (MO-M16) were shown to have the highest activity of lipase, protease, Mnp, and LiP, respectively. No yeast had amylase, cellulose, phytase, or laccase activity. Although only 60 isolates produced ligninolytic enzymes, 249 of the 257 yeasts could decolorize different dyes through the mechanism of biodegradation (222 isolates) or bio-sorption. The types of extracellular enzymes that the yeasts produced were significantly shaped by the types of wastewater treated.

1. Introduction

Yeasts, as a group of single-celled fungi, have been unequivocally recognized as having important roles in biotechnological applications such as fermentation, food, pharmacy, and fine chemicals (Kurtzman et al., 2011). The potential application of yeasts in wastewater treatment was suggested about 15 years ago by some Japanese scientists (Chigusa et al., 1996). More recently, some yeasts were revealed to be capable of producing lipase or of degrading phenol compounds, and thus have potential value in treating wastewater from oil manufacturing (Zheng et al., 2001), olive mill wastewater (Goncalves et al., 2009) or reclaiming oil-contaminated sites (Hesham et al., 2006). Our previous studies also demonstrated

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that yeasts could be advantageous in treating fermentation wastewater, paper-making wastewater, and dye-containing wastewater under the conditions of lab-scale reactors due to their high endurance to low pH, high salinity, and high chemical oxygen demand (COD) (Yang et al., 2003a, 2005a). However, the applications of such degrading yeasts in wastewater treatment rest on whether they can survive in an activated sludge system, especially in a system running for a long time. Unfortunately, yeast ecology in wastewater treatment systems has long been neglected compared to bacteria or other microbial populations, possibly because yeasts constitute only a minor fraction of the microorganisms present in activated sludge. Therefore, investigating the natural existence and ecological roles of yeasts in various existing full-scale wastewater treatment systems will be helpful for our understanding the yeast ecology, but also will provide important information for application of yeast technologies in wastewater treatment.

Few studies have investigated how yeasts are distributed in different wastewater treatment systems and what functions they perform. Our recent study results indicated that wastewater treatment systems harbor diverse yeast species compared to other environments, although yeasts are still a minor part of microorganisms present in activated sludge, and many yeast species were specific to a given wastewater treatment plant forming unique communities driven primarily by the type of influent processed and also the treatment process (Yang et al., 2011). This suggests that diverse yeast populations should have diverse functions to support their persistence in different wastewater treatment systems.

Yeasts could produce some important industrial enzymes, such as lipase, phytase, and protease, and some oxidase or peroxidase enzymes that could degrade aromatic substances. These extracellular enzymes play important roles in degrading large molecular pollutants in wastewater treatment. In this study, yeast isolates were cultivated from five different full-scale wastewater treatment plants. Their extracellular enzyme production was detected to evaluate the possible functions of yeasts in various wastewater treatment sludge systems. Since the yeast isolates were suitable to wastewater environments, the research results will inform new applications of wastewater and yeasts to produce industrially important enzymes.

2. Methods

2.1. Dyes and chemicals

Reactive Black 5 (Color Index), Reactive Brilliant Blue KNR (an anthraquinone dye) and Reactive Red M-3BE were from a commercial plant in Xinxiang, China. Reactive Black 5 and Reactive Red M-3BE are reactive azo dyes that are widely used in the textile industry. The stock solutions of each dye were prepared as 10 g l^{-1} and were diluted before use. Sterilization was achieved by membrane filtration (pore diameter, 0.45 μm).

2.2. Collection of wastewater treatment plant samples

Activated sludge samples were collected from five different full-scale wastewater treatment plants: an inosine fermentation plant (BP), a municipal wastewater treatment plant (M), a papermaking wastewater treatment plant (P), an antibiotic fermentation wastewater treatment plant (A), and a printing and dyeing wastewater treatment plant (D). The five plants were located in XinXiang, China and were running stably during the sampling period (May, 2008 for plant BP; June, 2008 for plant M; October, 2008 for plant P; March, 2009 for plant A, and March, 2009 for plant D).

Sludge samples were collected from the biochemical treatment units in the plants and were placed in sterile polypropylene tubes. Yeast isolation was conducted immediately after transport to the laboratory.

2.3. Isolation and identification of yeasts

Two culture media, YEPD and Martin's agar, were used to isolate yeasts (Jespersen et al., 2005). The homogenized samples were spread at different serial dilutions in triplicate on the media. After incubation at 28 °C for 3–5 days, yeasts were selected based on colony color, morphology, and cell characteristics under microscopic examination. The selected colonies were purified at least three times by sub-culturing on fresh agar plates. All yeast isolates were preserved on YEPD agar slants at 4 °C.

Genomic DNA was extracted from each yeast isolate was conducted using the cyltrimethylammonium bromide method (Fujita et al., 2001). The yield and fragmentation of the crude or purified DNA were determined by agarose gel electrophoresis (1% w/v agarose) and ultraviolet (UV) visualization after ethidium bromide (EB) staining. Purified DNA was used for 26S rRNA gene amplification.

The primer pair NL1 (5-GCA TAT CAA TAA GCG GAG GAA AAG-3) and NL4 (5-GGT CCG TGT TTC AAG ACG G-3) was used for the amplification of the D1/D2 domain of the 26S rRNA gene (O'Donnell, 1993). PCR was conducted using a 5 min initial denaturation at 95 °C, followed by 36 cycles of 60 s denaturation at 94 °C, 60 s primer annealing at 52 °C, 80 s extension at 72 °C, and a final 10 min extension at 72 °C (Guo et al., 2000). The PCR products were sent to Sangon Biotech (Shanghai, China) for sequencing on a PRISM 3700 DNA Analyzer (Applied Biosystems, Franklin Lakes, NJ).

All the sequences were submitted to Genbank (accession numbers in Fig. 4) and analyzed through a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) against the non-redundant nucleotide (nt) database. A phylogenetic tree was constructed using the neighbor-joining method in MEGA version 4.1. Yeast species were identified based on the phylogenetic analysis results (Kurtzman et al., 2011).

2.4. Detection of extracellular hydrolytic enzymes

Amylase, lipase, protease, and phytase were detected first by observing the colony morphology and clear zones on the starch, oil, milk, and phytic acid medium agar plates, respectively (Johnson and Case, 1995; Chi et al., 2003; Gupta et al., 2003; Haefner et al., 2005). Each selected isolate was inoculated in the four respective agar media and cultivated at 28 °C, pH 5–6 for 3–5 days. Yeast isolates producing extra-cellular amylase, protease, and phytase will form clear zones around the colonies due to the hydrolysis of polysaccharide, protein, and phytic acid, respectively, whereas isolates producing extracellular lipase will form crimson dots around the colonies due to the production of fatty acid and decreased pH. Cellulose was measured in liquid medium containing filter paper to observe the degradation of filter paper (Chi et al., 2009). The yeast isolate that displayed a positive reaction in corresponding enzyme detection was further confirmed and enzyme activity was measured in liquid medium under shaking conditions.

Activity of lipase was measured using a previously described titrimetric method (Watanabe et al., 1977). Protease activities in the liquid medium were assayed according to the Folin method (Kunamneni et al., 2003). One unit of lipase or protease is defined as the amount of enzyme necessary to produce 1 μmol of product per minute.

2.5. Ligninolytic enzymes assays

Supernatant (12,000*g* for 15 min) from yeast cultures was used for the following enzyme assays. Lignin peroxidase (LiP) activities