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Evaluation of cell disruption method for lipase extraction from novel thraustochytrids

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ABSTRACT

Marine microorganisms represent a rich source of enzymes with the potential industrial application. In this study, we screened novel thraustochytrids for a range of enzymatic activities. The similarity between isolates based on enzyme activities was investigated using cluster analysis. Thraustochytrids were observed to possess different enzymatic activities, however, we focused on lipase activity. Two strains AMCQ-4b27 (34 IU/g) and AMCQS1-9 (36 IU/g) exhibited highest intracellular lipase activity among the isolates. To extract lipase, four different cell disruption methods such as bead vortexing, grinding, sonication and homogenization, were evaluated for their efficiency on lipase extraction yields and quality. Sonication was found to be the best method for enhancing lipase extraction yields from *Thraustochytrium* sp. AMCQ-4b27 (0.903 IU/μg) and *Schizochytrium* AMCQS1-9 (1.44 IU/μg).

1. Introduction

The marine environment has a unique biodiversity and is a good source of enzymes with unusual properties with potential utility in biotechnology [1]. Considerable effort has gone into screening extracts from marine organisms for novel bioactive compounds with unique structural classes [2,3]. Marine microorganisms are particularly useful for bioactive compounds discovery since they can be grown in a controlled environment and offer ease of scale up compared to other natural sources [4]. Among marine microbes, thraustochytrids are one of the most scaleable organisms since they are commercially useful for the production of omega-3 oils and carotenoids [5]. Thraustochytrids can accumulate approximately 40% docosahexaenoic acid (DHA) as a percentage of total lipids [6,7]. Thraustochytrids have been used as animal feed, aquaculture feed and poultry feed in addition to their use in omega-3 and carotenoid production [8,9]. These heterotrophic microorganisms also are a source of enzymes with potential application in food industry [10,11]. Thraustochytrids have been reported to produce degradative enzymes such as lipases, peptidases, phosphatases and cellulases, although the utility of their enzymes has not been fully investigated [12,13].

Enzymes obtained from marine microbes possess a wide range of applications such as bioconversions including organic synthesis, and in food, detergent, paper, oleochemical industries, cosmetics, medicine and waste treatment [14]. Some of the enzymes are secreted in the medium and can be easily accessed. In contrast, recovery of intracellular enzymes often requires complicated downstream process [15]. The recovery of specific intracellular components from microalgae are hindered by a rigid cell wall, thus requires an additional cell disruption step to facilitate the release of intracellular components. Cell disruption is an important downstream processing step as it impacts extraction yields and therefore the cost of production of bioactive compound [16]. Various physical, chemical and mechanical methods are available for disrupting microbial cells, mechanical methods generally used for large-scale processing [15]. The selection of a suitable method of cell disruption for extracting intracellular products depends on cell wall strength, intracellular location of products, stability and the final use of the recovered products [17,18]. For example, the effect of chemical and mechanical cell disruption methods was investigated for extraction of intracellular β-D-galactosidase from *Kluyveromyces lactis* [17].

Lipases are hydrolytic enzymes that catalyse the hydrolysis or synthesis of esters and possess positional as well as fatty acid selectivity [19,20]. Lipases isolated from marine organisms generally showed maximum activity at low temperatures and have unusual fatty acid specificity [3]. Lie and Lambertsen observed that the crude enzyme mixture collected from cod intestine showed an unusual fatty acid selectivity, preferentially hydrolysed omega-3 fatty acids [21]. Similar fatty acid selectivity was also observed from the crude digestive juice

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from salmon and rainbow trout collected from the *pyloric* area of the stomach [22]. However, in each of these cases no omega-3 specific lipase has been characterised. A recent study reported that a novel lipase isolated from the marine microbe *Marinobacter lipolyticus* SM19 showed a high preference for hydrolysing eicosapentaenoic acid (EPA) from fish oil [23].

We report here the efficiency of various cell disruption methods for release of intracellular lipases from thraustochytrids. The efficiency of a method was investigated based on the release of intracellular proteins and their lipolytic activity. To the best of our knowledge this is the first report on optimisation of cell disruption methods for lipase extraction from thraustochytrids.

2. Materials and methods

2.1. Chemicals

All chemicals including *p*-nitrophenyl palmitate (pNPP), Tween 80, and medium components (yeast extract and peptone) were procured from Sigma-Aldrich, USA, acid washed glass beads from Thomas Scientific, Australia and Sea salts from Instant Ocean, USA. The API ZYM kit was procured from BioMérieux, Australia Pty. Ltd.

2.2. Strain selection and cultivation

Recently isolated strains designated as AMCQS5-3 (Genbank accession numbers JX993839), AMCQS5-4 (Genbank accession numbers JX993840), AMCQS5-5 (Genbank accession numbers JX993841), AMCQS1-9 (Genbank accession numbers JX993843) and AMCQ-4b27 (Genbank accession numbers JX993842) were isolated from the Queenscliff region, Victoria, Australia (during February 2011) and used for screening lipase activity [24]. Two microbial cultures, *Schizochytrium* S31 (S31) and PRA 296, were procured from the American Type Culture Collection (ATCC).

Thraustochytrids were maintained in a GYP medium containing glucose (5 g L^{-1}), peptone (2 g L^{-1}) and yeast extract (2 g L^{-1}) and artificial sea water (50% v/v) at 20 °C. Stock cultures were subcultured onto fresh plates at regular intervals. All thraustochytrids were grown in modified Vishniac's Medium (0.1% glucose, 0.01% yeast extract, 0.01% peptone, 0.1% gelatine hydrolysate, 1.2% agar in seawater, pH 7.0) for enzyme screening [25]. The incubation conditions were 20 °C and 150 rpm agitation until the cells reach the late exponential growth phase (96 h). Before harvesting, cells were observed under a microscope for integrity and growth.

2.3. Enzymatic profiling of isolates and cluster analysis

The thraustochytrid cells were harvested at 5000 rpm for 15 min and washed with sterile artificial sea water. Cells were suspended in artificial sea water and 65 μL of cell suspension (corresponds to 4.0×10^5 cells) added into (19 microwells except in control) API ZYM strips and incubated at 20 °C overnight. After incubation, 30 μL of API ZYM A and B were added to the microwells and incubated for 5 min. Enzyme activities were measured using a colorimetric scale and experiments were conducted in triplicate [26].

Enzyme cluster analysis was performed using XLSTAT Version 2014.5 based on Agglomerative hierarchical clustering (AHC) method. This method helps in analysing understanding the similarity between thraustochytrids isolates based on enzymatic activities.

2.4. Lipase production

Thraustochytrid cells were grown in medium containing peptone (500 mg L^{-1}), yeast extract (500 mg L^{-1}) and Tween 80 (1% v/v) [27]. The production medium was pre-adjusted to pH 6.0 using 0.1 M HCl. A volume of 50 mL medium in a 250 mL Erlenmeyer flask was inoculated with a loop full of culture from the plate. The flask was incubated at 20 °C with shaking at 150 rpm for 48 h. Inoculum (5% v/v) was used for the production of biomass. The production culture was incubated for 120 h under the same conditions. The biomass was harvested by centrifugation (5000 rpm, 10 min). Harvested biomass was resuspended in distilled water to remove the Tween 80, and this process was repeated three times [28]. The biomass was kept at − 80 °C overnight and freeze-dried (Freeze dryer, Christ, Germany) for 24 h for further use.

2.5. Cell disruption methods for lipase release

2.5.1. Bead vortexing

A sample of freeze-dried biomass (100 mg) was suspended in 1 mL of extraction buffer (100 mM Tris pH 7.2) with EDTA (10 mM) and NaCl (100 mM). One milliliter of acid washed glass beads (425–600 μm, Klausen Pty Ltd., Australia) was added and the contents were vortexed for 10 min, in 30 s bursts. Ground biomass was centrifuged at 12,000 rpm for 60 min at 4 °C. The supernatant was analysed for lipase activity and protein levels as described below.

2.5.2. Grinding with liquid nitrogen

A sample of freeze-dried biomass (100 mg) was suspended in 10–15 mL of liquid nitrogen and ground with a pestle for 2 min. 1 mL of extraction buffer was added and the resultant biomass was collected and centrifuged at 12,000 rpm for 60 min at 4 °C. The supernatant was analysed for lipase activity and protein levels.

2.5.3. Sonication

A sample of freeze-dried biomass (100 mg) was suspended in 1 mL of extraction buffer. The suspension was sonicated at 20 kHz, 40% amplitude and the pulse was 40 s on 20 s off through a time-range of 5–25 min (Sonics, USA). After every 5 min, cells were observed under a microscope to check the extent of disruption. During sonication, the sampling tubes were kept in ice-bath to avoid heat-mediated denaturation of crude enzyme. The suspension was centrifuged at 12,000 rpm for 60 min at 4 °C. The supernatant was analysed for lipase activity and protein concentration.

2.5.4. Homogenization

Homogenization was performed using a rotor-stator type homogenizer (Unidrive 1000, CAT Scientific, USA). A sample of freeze-dried biomass (100 mg) was suspended in 1 mL of extraction buffer and the suspension was subjected to homogenization at 8500 rpm/min for 5–25 min at 5 min intervals. After every 5 min, the suspension was observed under the microscope. The suspension was centrifuged at 12,000 rpm for 60 min at 4 °C. The supernatant was analysed for protein concentration and lipase activity.

All the cell disruption methods for protein release were conducted in four replicates and the error bars shown in the figures represent the standard error. The relative extraction efficiency of the cell disruption methods was calculated relative to the highest protein yield and expressed as fractions thereof.

2.6. Microscopic observation of the native and disrupted thraustochytrid cells

The cell slurries were observed under a microscope using differential interference contrast (Axio-imager, Zeiss, Germany) to check for disruption. Smears of normal and disrupted cells were prepared on the glass slide and air-dried for examination.

2.7. Lipase and protein assay

Lipase activity was measured from the supernatant obtained after the cell disruption using *p*-nitrophenyl palmitate (pNPP) as a substrate,