NEW MECHANICAL DISRUPTION METHOD FOR EXTRACTION OF WHOLE CELL PROTEIN FROM *CANDIDA ALBICANS*

SF Wong, JW Mak and PCK Pook

International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia

Abstract. Cell disruption or lysis is a crucial step to obtain cellular components for various biological studies. We subjected different concentrations of *Candida albicans* to 5, 10, 15 and 20 cycles of disruption. The degree of cell lysis was observed using light microscopy and the yields obtained were measured and analysed. The optimum extraction with 1 x 10^{10} yeast cells/ml was achieved after 5 cycles of disruption with 1.0 mm diameter glass beads at 5,000 rpm. Approximately 80% of the cells were lysed and the protein yield was 6,000 μg/ml. SDS-PAGE analysis revealed approximately 25 distinct protein bands with molecular weights ranging from 8 kDa to 220 kDa. We conclude that this mechanical disruption of fungal cells is a rapid, efficient and inexpensive technique for extracting whole cell proteins from yeast cells.

INTRODUCTION

Cellular disruption of fungi is a crucial step in obtaining maximum amounts of soluble cellular contents with maximum biological activity and with minimum denaturation, proteolysis and oxidation. The fungal cell wall is extremely difficult to disrupt because of its complex nature compared with mammalian cell membrane. The cell wall of *Candida albicans* is a multilayered structure located externally to the plasma membrane and while providing rigidity to the cell, acts as a permeable barrier, which protects the protoplast against physical and osmotic injury. The major components (80 to 90%) of the cell wall are carbohydrates and consist of (1) mannan or polymers of mannose covalently associated with proteins to form glycoproteins (mannoproteins) (2) β-glucans, which are branched polymers of glucose containing β-1, 3 and β-1, 6 linkages, and (3) chitin, which is an unbranched homopolymer of N-acetyl-D-glucosamine containing β-1, 4 bonds (Chaffin *et al*, 1998; Martinez *et al*, 1998).

Cell disruption technology can be divided into mechanical and non-mechanical methods. Mechanical methods include ultrasonic disruption (sonicator), mechanical agitation (homogenizer, mortar and pestle, blender) and pressurized disruption (French press). The non-mechanical methods consist of chemical permeabilization (cationic/anionic detergents), physical disruption (osmotic shock, pressure release, freezing and thawing) and enzymatic permeabilization (lyticase, zymolyase, proteinase K).

To our knowledge, there is no report on an efficient mechanical method for fungal cell wall disruption for optimum extraction of fungal whole cell protein. In this study, we report a new mechanical method we developed to increase the efficiency of fungal whole cell protein extraction.

MATERIALS AND METHODS

Culturing of *Candida albicans*

C. albicans was grown in YPD medium (1% yeast extract, 2% bacteriological peptone and 2% D-glucose) at 37ºC for 72 hours on a

Correspondence: Wong Shew Fung, International Medical University, Bukit Jalil, 57000 Kuala Lumpur, Malaysia.
Tel: 603-86567228 ext 1003; Fax: 603-86567229
E-mail: shewfung_wong@imu.edu.my

shaking incubator (Certomat SII and Certomat H, B Braun Biotech International, GmbH, Melsungen, Germany). The yeast cells were harvested, washed with sterile phosphate-buffered saline (PBS) three times by sedimenting at 2,000 rpm for 10 minutes and then re-suspended in sterile PBS. The number of yeast cells was determined with a hemocytometer and adjusted to the desired concentrations (10^6, 10^8 and 10^{10} yeast cells/ml) for the cell disruption experiment.

Cell disruption

Polystylene sample tubes (2.0 ml, Tomy Degital Biology) were used throughout the experiment. Approximately 0.7 to 0.8 g of 1.0 mm diameter glass beads (Tomy Degital Biology) and 1 ml aliquot of each concentration of *C. albicans* was added into each tube. The samples were cooled at -80ºC for 60 seconds before being subjected to disruption with a Micro Smash™ beads cell disrupter/micro homogenizing system (MS-100, Tomy Degital Biology) for 120 seconds at 5,000 rpm. The samples were then cooled at -80ºC for 5 minutes and the disruption cycle was repeated. Representative samples after 5, 10, 15 and 20 disruption cycles were obtained for protein concentration determination, sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and microscopy. The tests were conducted in duplicate.

Determination of protein concentration

Concentrations of the lysates were determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) based on the method of Bradford (1976). The absorbance was measured at 595 nm with a spectrophotometer (Spectro UV-Vis Auto, LaboMed, Culver City, USA), and the protein concentration was determined from a standard curve using bovine serum albumin as standard protein.

SDS-PAGE

Protein samples were denatured by boiling at 90ºC for 10 minutes in Laemmli sample buffer (Bio-Rad) containing 31.25 mM Tris-HCl (pH 6.8), 12.5% glycerol, 1% SDS, 0.005% bromophenol blue and 2.5% β-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA). Samples were centrifuged at 12,000*g* for 5 minutes to prevent streaking during electrophoresis and kept on ice before loading into the wells.

Samples were separated in 12.5% (w/v) polyacrylamide gel using the discontinuous buffer system of Laemmli (1970) using TV400 Maxi (20 x 20 cm) vertical electrophoresis unit (Scie-Plas, UK). Electrophoresis was performed at room temperature at a constant voltage of 120V for stacking gel and 150V for resolving of polypeptides. Gel was stained with SimplyBlue SafeStain (Invitrogen Corporation, California) and photographed.

The relative molecular weights of the polypeptide bands were estimated by comparing their electrophoretic mobilities with those of known molecular weights of prestained SDS-PAGE standards (Sigma ColorBurst Electrophoresis Markers). The standard molecular weight markers used were: violet, 220,000 Da; pink, 100,000 Da; blue, 60,000 Da; pink, 45,000 Da; orange, 30,000 Da; Blue, 20,000 Da, pink, 12,000 Da and blue, 8,000 Da.

Microscopy examination

Undisrupted and disrupted yeast samples were examined under 400x magnification using a Leica DMLS compound microscope and the degree of fungal lysis was assessed visually. The images were captured using a 5.1 megapixal digital camera and documented using an image pro software.

RESULTS

Determination of protein concentration

To assess the efficiency of this method of cell disruption, we subjected different concentrations of *C. albicans* to various disruption cycles and the total protein concentra-

tions were determined (Table 1). The total protein concentrations obtained ranged from 13 to 37 μg/ml, 100 to 188 μg/ml and 6,232 to 6,413 μg/ml for 1 x 10^6, 1 x 10^8 and 1 x 10^{10} yeast cells/ml, respectively. The total protein obtained for 1 x 10^6 yeast cells/ml increased slightly as the number of disruption cycles increased from 5 to 20 cycles. This trend was not observed for samples of 1 x 10^8 and 1 x 10^{10} yeast cells/ml. The protein yields were similar after 5, 10, 15 and 20 disruption cycles.

Protein banding patterns of *C. albicans* following disruption

SDS-PAGE analysis of 1 x 10^6 (Fig 1, lanes 2-5), 1 x 10^8 (Fig 1, lanes 6 - 9) and 1 x 10^{10} (Fig 1, lanes 10-13) *C. albicans* yeast cells per ml after 5 disruption cycles (Fig 1, lanes 2, 6 and 10), 10 cycles (Fig 1, lanes 3, 7 and 11), 15 cycles (Fig 1, lanes 4, 8 and 12) and 20 cycles (Fig 1, lanes 5, 9 and 13) of disruption showed no protein bands on the gel for the 1 x 10^6 yeast cells/ml. There were only two to three light bands observed for the 1 x 10^8 yeast cells/ml sample. SDS-PAGE analysis showed approximately 25 distinct protein bands with molecular weight ranging from less than 8 kDa to more than 220 kDa for samples of 1 x 10^{10} yeast cells/ml.

Table 1
Total protein concentrations of *C. albicans* after mechanical disruption. Values are expressed as means of triplicate determinations plus 1 SD.

No. of yeast cells/ml	No. of disruption cycle	Total protein concentration (μg/ml)
1 x 10^6	5	13.8 ± 0.9
	10	21.7 ± 4.0
	15	29.7 ± 2.4
	20	37.2 ± 3.1
1 x 10^8	5	151.1 ± 29.9
	10	100.3 ± 19.6
	15	147.6 ± 13.0
	20	188.8 ± 8.8
1 x 10^{10}	5	6,368.9 ± 287.1
	10	6,392.0 ± 265.3
	15	6,413.3 ± 269.4
	20	6,232.5 ± 258.1

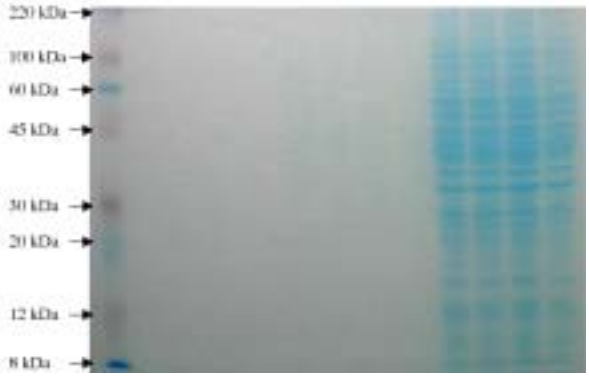


Fig 1–Protein bands patterns of 1 x 10^6 (lanes 2-5), 1 x 10^8 (lanes 6 - 9) and 1 x 10^{10} (lanes 10-13) *C. albicans* yeast cells per ml after being subjected to 5 cycles (lanes 2, 6 and 10), 10 cycles (lanes 3, 7 and 11), 15 cycles (lanes 4, 8 and 12) and 20 cycles (lanes 5, 9 and 13) of disruption.

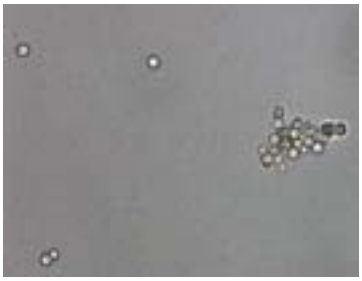
Microscopic analysis of fungal cell wall disruption

The degree of fungal cell lysis seen after 5 (Fig 2b), 10 (Fig 2c), 15 (Fig 2d) and 20 (Fig 2e) cycles of disruption were assessed visually under 400x magnification using untreated culture (Fig 2a) as controls. From 80 to 90% of the yeast cells were lysed after being subjected to the first 5 cycles of disruption. Cells were broken into smaller fragments after being subjected to further disruption cycles.

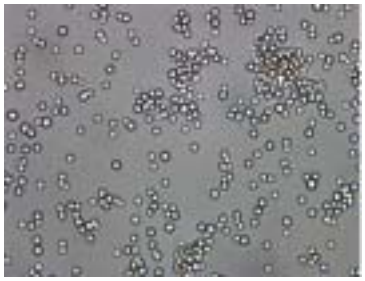
DISCUSSION

In the present study, an efficient and inexpensive mechanical method was developed for the disruption of *C. albicans*. This method utilized the shearing force generated by 3-D rotating high speed motion between glass beads and the cells. The disruption of yeast

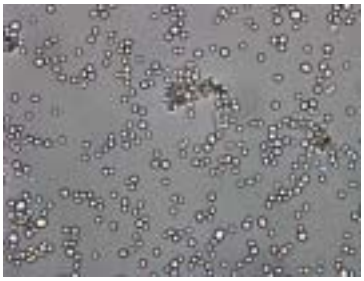
(a) Before disruption



1×10^6



1×10^8



1×10^{10} (1: 100 dilution)

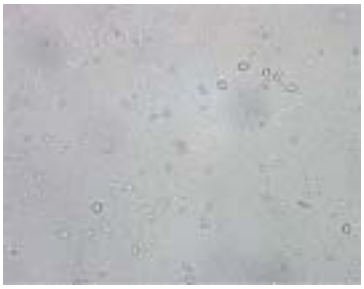
(b) 5 cycles

1×10^6

1×10^8

1×10^{10} (1: 100 dilution)

(c) 10 cycles



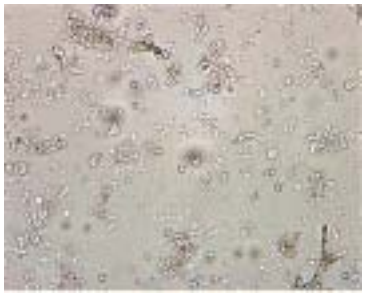
1×10^6

1×10^8

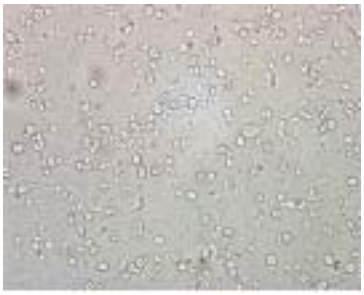
1×10^{10} (1: 100 dilution)

(d) 15 cycles

1×10^6

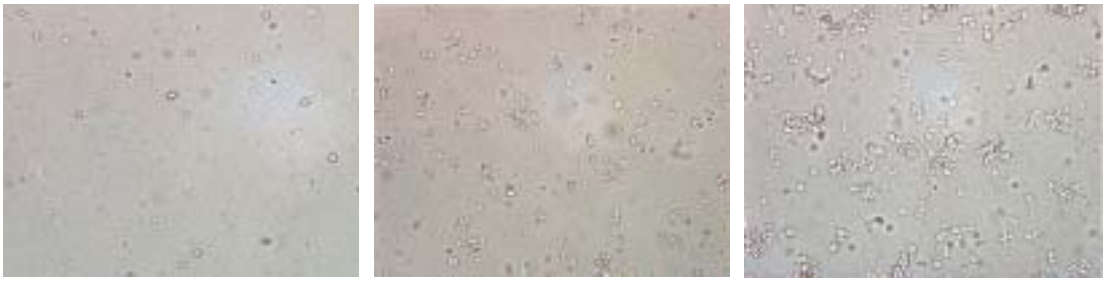


1×10^8



1×10^{10} (1: 100 dilution)

(e) 20 cycles



1 x 10^6 (1:10 dilution) 1 x 10^8 (1:10 dilution) 1 x 10^{10} (1: 100 dilution)

Fig 2–Microscopic examination of *C. albicans* (a) before disruption and after being subjected to (b) 5 cycles, (c) 10 cycles, (d) 15 cycles and (e) 20 cycles of disruption.

cells was caused by the grinding between and collision with the beads. The size of the glass beads may determine the efficiency of disruption. We recommend the use of glass beads 1.0 mm in diameter as the optimum bead size for this application after testing with 0.1, 0.5 and 1.0 mm glass beads (data not shown). The loading of the beads should be approximately 50% of the total liquid biomass volume (approximately 0.8 g in 1 ml of yeast cells) and the total weight of each tube and sample should not exceed 5 g. After the disruption, the beads settled by gravity in seconds and the cell extract was easily recovered for further usage.

Manning and Mitchell (1980a, b) used Braun MSK cell homogenizer (B Braun Biotech, Melsungen, Germany) for the preparation of cytoplasmic extracts from *C. albicans*. The cells were broken with 0.45 mm diameter glass beads in this cell homogenizer for 90 to 120 seconds under pulses of liquid carbon dioxide for cooling. This homogenizer disrupts sample sizes up to 40 ml and relies on cooling with liquid CO_2 to keep the samples at an acceptable temperature. This method is more costly and may require prior sample preparation with lysis buffer (Pitarch *et al*, 2002) before being lysed mechanically with this homogenizer, vortex (Pitarch *et al*, 1999; Niimi *et al*, 1996) or FP 120 FastPrep cell disrupter (Bio 101, Inc) (Muller *et al*, 1998).

Some researchers utilized the French press to disrupt *C. albicans* yeast cells and germ tubes by two consecutive preparations in a French pressure cell at 20,000 lb/in^2 (Trinel *et al*, 1993). The French press technique is less time consuming than other methods such as sonication. The biochemical integrity of the sample is maintained as there is less heat generation. There is nearly complete breakage of the cells and minimum sample loss compared with other methods such as grinding. The drawback is the cost of the equipment. The ultrasonic cell disrupter typically uses half wavelength probes about 15 cm long that operates at 20, 40 or 1 MHz to obtain rapid cell disruption (Borthwick *et al*, 2005). Sixteen medically important fungal species (*Aspergillus* spp, *Bipolaris* spp, *Candida albicans*, *Exserohilum longirostratum*, *Fusarium solani*, *Paeciliomycesm marquandii*, *Penicillium chrysogenum*, *Scedosporium apiospermum* and *Trichophyton mentagrophytess*) were sonicated on ice for 90 seconds at 150 Hz using a Labsonic U sonicator (B Braun, Germany) (Karakousis *et al*, 2006). Only 25 to 50% of *C. albicans* were lysed compared to control cultures. We had previously used this method to lyse *C. albicans* mechanically without the addition of any enzyme or lysis buffer. We found approximately 50% of the yeast cells

were lysed with a similar sonicator after 20 cycles of disruption with multiple freezing in liquid nitrogen and thawing in between each cycle (data not shown). The disadvantages of this method may include heat generation, high noise level and yield variability.

In another study, the yeast cell pellets were rapidly frozen in liquid nitrogen and then mechanically broken with an electric coffee grinder using dry ice as coolant (Schultz *et al*, 1997; Singleton *et al*, 2001) or with a mortar and pestle (Schultz *et al*, 1991, 1997; Karakousis *et al*, 2006). Caution must be taken when using liquid nitrogen.

A number of researchers used enzymatic methods for the disruption of yeast cells for their studies (Sundstrom and Kenny, 1985; Lopez-Ribot *et al*, 1991; Marot-Leblond *et al*, 1995; Mizutani *et al*, 2000; Karakousis *et al*, 2006). The fungi were digested with 1 mg/ml of proteinase K, 400 U lyticase or zymolase. When the yeast cells were treated with these lytic enzymes in the presence of osmotic stabilizers, the cell walls of fungi were removed giving rise to the formation of spheroblasts. The removal of the wall of the yeast cells may destroy antigenic sites and render the protein extract unsuitable for immunological studies such as the generation of monoclonal or polyclonal antibodies.

In this study, we found the optimum concentration of yeast cells for disruption to obtain maximum protein yields was at 1 x 10^{10} yeast cells/ml. The yields increased 32 to 63-fold as the concentration of yeast cells increased by 100-fold from 1 x 10^{8} yeast cells/ml. This was clearly revealed on the SDS-PAGE where no or only 3 to 4 protein bands were detected at the lower concentration of yeast cells. At 1 x 10^{10} yeast cells/ml, approximately 25 distinct protein bands with molecular weight ranging from less than 8 kDa to more than 220 kDa were detected.

In summary, this mechanical method was effective and convenient for the preparation of whole cell protein extraction at laboratory scale for further applications. Our protocol was reproducible and generated good yields with maximum cell disruption without the addition of any chemicals or enzymes. This will ensure that the maximum amount of cellular constituents is solubilized while still maintaining maximum biological activity of the released products.

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