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Whole cell immobilisation of *Natrinema gari* BCC 24369 for histamine degradation

Wanaporn Tapingkae [a], Kirk L. Parkin [b], Somboon Tanasupawat [c], Jittiporn Kruenate [d], Soottawat Benjakul [a], Wonnop Visessanguan [e,*]

[a] Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[b] Department of Food Science, University of Wisconsin, Madison, WI 53706, USA
[c] Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand
[d] National Metal and Materials Technology Center (MTEC), 114 Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand
[e] National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand

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ABSTRACT

Whole cells of *Natrinema gari* BCC 24369, a novel halophilic archaeon, were immobilised on various matrices by different techniques. Among all matrices tested, Celite showed the highest immobilisation yield. The immobilised whole cells retained histamine-degrading activity as high as 94% of the original activity detected in free whole cells. The catalytic properties of the immobilised whole cells on the Celite support were similar to the corresponding free whole cells, including optimal NaCl concentration (4.0–5.0 M), optimal pH (6.5–7.5), and optimal temperature (40–55 °C). Histamine-degrading activity, either in the presence of NaCl at elevated concentrations or at elevated temperatures, became more stable due to immobilisation. Both free and immobilised whole cells were able to degrade histamine specifically. The immobilised whole cells could be reused for the degradation of histamine up to seven cycles without any significant loss in activity. The immobilised whole cells have the potential to be applied for the degradation of histamine in products high in salt.

1. Introduction

The presence of high levels of histamine in seafoods and seafood products may cause the rejection for import and is also associated with the food poisoning. Histamine is heat stable and not detectable through organoleptic analysis, even by trained panellists (Arnold, Price, & Brown, 1980). Except for gamma irradiation, no other food processing methods are available for histamine degradation (Etkind, Wilson, Gallagher, & Cournoyer, 1987; Kim et al., 2004).

Natrinema gari BCC 24369 is a novel halophilic archaeon (Tapingkae et al., 2008) that can reduce histamine in culture broth during cultivation in the presence of 4.3 M NaCl. The histamine-degrading activity was mediated through the intracellular enzyme and needed an electron acceptor for its activity (Tapingkae, 2009). Due to the limitations of applying *Nmn. gari* BCC 24369 cells and its enzyme caused by the slow growth rate, low yield of enzyme, and the cost of cell or enzyme preparation, the immobilisation of whole cells might provide potential advantages over applications of free cells and enzyme systems.

Cell immobilisation is the physical confinement or localisation of intact cells to a certain defined region of space with preservation of some desired catalytic activity (Karel, Libicki, & Robertson, 1985). The use of immobilised whole microbial cells and/or organelles eliminates the often tedious, time-consuming, and expensive steps involved in isolation and purification of intracellular enzymes (Ohmiya, Ohashi, Kobayashi, & Shimizu, 1977). Additionally, the stability of enzyme can be enhanced by retaining its natural catalytic surroundings during immobilisation and subsequent continuous operation (Karel et al., 1985). Furthermore, metabolically active cell immobilisation is particularly preferred where co-factor regeneration machinery is necessary for the catalytic reaction (Bernal, Sevilla, Cánovas, & Iborra, 2007).

Immobilisation is commonly accomplished by using four techniques, physical entrapment within a porous matrix, attachment or adsorption to a pre-formed carrier, self-aggregation by flocculation and cell contained behind barrier (Pilkington, Margaritis, Mensour, & Russell, 1998). Nevertheless, a few critical parameters such as the cost of immobilisation, mass transport limitations and applicability to a specific end-product should be taken into consideration. To maximise the use of whole cells of the *Nmn. gari* BCC 24369 for degradation of histamine in the presence of high salt concentrations without cell disintegration and complex purification of the enzyme, the appropriate immobilisation technique should be applied. The objectives of this study were to select the most effective immobilisation method for whole cells of *Nmn. gari* BCC 24369 and to study the properties of both free and immobilised whole cells.

* Corresponding author. Tel.: +66 2564 6700x3747; fax: +66 2564 6590.
E-mail address: wonnop@biotec.or.th (W. Visessanguan).

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2. Materials and methods

2.1. Culture and growth condition for Nmn. gari BCC 24369

Nmn. gari BCC 24369 was grown on an agar plate of halophilic medium (pH 7.2). The medium (1 l) contained 250 g NaCl, 5 g casamino acid, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4 \cdot 7H_2O$, 0.036 g $FeCl_2 \cdot 4H_2O$, 0.00036 g $MnCl_2 \cdot 4H_2O$ and 20 g agar. The inoculum was prepared by inoculating a loopful of culture into 5 ml of halophilic broth and incubating at 37 °C in a shaker incubator (Sartorius, Certomat® BS-1, Goettingen, Germany) at 200 rpm for 7 days. Cells were cultivated by inoculating 5% (v/v) of seed culture into 200 ml of halophilic medium containing 500 ppm of histamine (free-base) in a 500-ml Erlenmeyer flask, and incubated at 37 °C in a shaker incubator at 200 rpm for 7 days. Cells were harvested in the exponential phase ($A_{600} \sim 5.0$) by centrifugation at 15,000*g* for 10 min and washed twice with 50 mM Tris–HCl buffer, pH 7.0 containing 4.3 M NaCl. The cell pellet was recovered by centrifugation at 15,000*g* for 10 min and referred to as wet cell paste.

2.2. Immobilisation of whole cells

2.2.1. Adsorption

Pig bone: The procedure of Negishi, Sato, Mukataka, and Takahashi (1989) was used with some modifications. Pig bone powder was washed with ethanol five times, followed by distilled water. Five grams of washed pig bone powder were added to 10 ml of 50 mM Tris–HCl buffer, pH 7.0, containing 4.5 M NaCl (referred to as standard buffer) and 1 g of wet cell paste. The mixture was agitated (100 rpm) for 1 h at 0–4 °C. The immobilised whole cells were filtered using Whatman paper No. 1, washed with a standard buffer until no protein was released, dried under vacuum for 3 h at room temperature and stored at 4 °C until used.

Chitosan flakes: The procedure of Pereira, Zanin, and Castro (2003) was applied with some modifications. Five grams of chitosan flakes (Bioshell Inc., OR) were soaked in hexane under agitation conditions (100 rpm) for 1 h, and air dried. The soaked chitosan flakes were added to 10 ml of standard buffer containing 1 g of wet cell paste. The mixture was agitated (100 rpm) for 3 h at room temperature, and allowed to stand for 18 h at 4 °C. The immobilised whole cells were filtered, washed, dried, and stored at 4 °C until used.

Celite: The procedure of Khare and Nakajima (2000) was used with some modifications. Celite® 545 (5 g) (Mallinckrodt Baker Inc., Phillipsburg, NJ) was added to 10 ml of standard buffer containing 1 g of wet cell paste. The mixture was agitated (100 rpm) for 1 h at 0–4 °C. The immobilised whole cells were filtered, washed, dried, and stored at 4 °C until used.

2.2.2. Entrapment

Agar: The procedure of Takeno, Yamaoka, and Sasaki (2005) was used with some modifications. Standard buffer containing 4% (w/v) agar (Difco; BD, Franklin Lakes, NJ) was autoclaved at 121 °C (15 psi) for 15 min. Warm agar solution (50 °C, 2.5 ml) was mixed with 2.5 ml of standard buffer containing 1 g of wet cell paste at 60 °C. This mixture was quickly poured into a petri dish to obtain a thickness of 8 mm and cooled at room temperature. The solidified agar was cut into 8 mm cubes ($\sim 8 \times 8 \times 8$ mm) and washed with standard buffer until no protein was released. The obtained immobilised whole cells were stored at 4 °C until used.

Ca^{2+}-alginate beads: The procedure of Takeno et al. (2005) was used with some modifications. A 2.5-ml aliquot of sodium alginate (Acros Organics, Fair Lawn, NJ) solution (3%, w/v) was mixed with 2.5 ml standard buffer containing 1 g whole cells. The slurry was added dropwise (diameter ~2 mm) into 100 ml of 0.15 M $CaCl_2$. After 30 min, the gel beads containing immobilised whole cells were washed and stored at 4 °C until used.

2.2.3. Cross-linking

Alginate–chitosan beads: The procedure of Vidyasagar, Prakash, and Sreeramulu (2006) was used with some modifications. A 2.5-ml aliquot of 3% (w/v) sodium alginate solution was mixed with 2.5 ml of standard buffer containing 1 g of wet cell paste. The slurry was added dropwise (diameter ~2 mm) into 100 ml of 0.5% (w/v) chitosan flakes dissolved in 3% (v/v) acetic acid solution containing 0.5% (w/v) $CaCl_2$. After 30 min, the gel beads containing immobilised whole cells were washed and stored at 4 °C until used.

Cross-linked alginate beads: The procedure of Vidyasagar et al. (2006) was used with some modifications. A 2-ml aliquot of 3% (w/v) sodium alginate solution was mixed with 2 ml of standard buffer containing 1 g of wet cell paste. One millilitre of glutaraldehyde solution (0.5%, v/v) was added, followed by gentle mixing. The mixture was kept for 90 min at 30 °C. The slurry was added dropwise (diameter ~2 mm) into 100 ml of 0.05 M $CaCl_2$. After 30 min, the gel beads containing immobilised whole cells were washed and stored at 4 °C until used.

Cross-linked chitosan beads: The procedure of Carrara and Rubiolo (1994) was used with some modifications. Chitosan (3 g) was dissolved in 100 ml of 2% (v/v) acetic acid with continuous stirring for 90 min. The chitosan solution was filtered through cheesecloth to remove insoluble materials and was added dropwise (diameter ~4 mm) into 150 ml of 1.5% (w/v) sodium triphosphate solution with gentle stirring. The chitosan beads were washed with standard buffer until neutrality. The beads were activated with standard buffer containing 1% (v/v) glutaraldehyde for 24 h at room temperature. A 5 g sample of the activated chitosan beads was mixed with 10 ml of standard buffer containing 1 g of wet cell paste. The mixture was gently agitated (100 rpm) at room temperature overnight. The beads obtained were washed and stored at 4 °C until used.

Eggshell: The procedure of Venkaiah and Kumar (1994) was used with some modifications. Small pieces of hen eggshell were boiled for 20 min, washed with acetone, and dried in an oven at 60 °C for 12 h. The pieces were crushed in a blender for 5 min and sieved through a 100 mesh. Ground eggshell (5 g) was mixed with 10 ml of standard buffer containing 1 g of wet cell paste. The mixture was gently agitated at 100 rpm and 2 ml of 6% (v/v) glutaraldehyde were slowly added during stirring. The mixture was incubated at 10 °C for 12 h. The residues were obtained by centrifugation at 4 °C, 6000*g* for 15 min. The immobilised whole cells were washed, dried, and stored at 4 °C until used.

2.3. Histamine-degrading activity assay and protein determination

The histamine-degrading activity was assayed using histamine as substrate. Unless otherwise stated, the free or immobilised whole cells (0.1 g) were added into 1 ml of standard assay mixture consisting of 50 mM Tris–HCl buffer, pH 7.0, 4.3 M NaCl, 500 μM 1-methoxy-5-methylphenazinium methylsulphate (1-methoxy PMS), and 5 mM histamine dihydrochloride. The reaction mixture was incubated at 37 °C for precisely 1 h under static conditions, unless otherwise stated. Enzymatic reaction was terminated by adding 1 ml of 0.1 N HCl. A blank analysis was performed in the same manner, except the free or immobilised whole cells were added after addition of 1 ml 0.1 N HCl. The histamine contents of reaction and blank were determined by the fluorometric method of AOAC. (1995). One unit of activity was defined as the degradation of 1 μmol histamine per hour per gram of matrix under the specified conditions.