MICROBIOLOGY INDONESIA, April 2007, p 48-50 Volume 1, Number 1
ISSN 1978-3477

Screening of Lactic Acid Bacteria for the Purpose of Chitin Recovery Processing

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Lactic acid fermentation has been studied as an alternative method of chitin recovery from the natural chitin compounds, such as shrimp waste. The purpose of this research was to identify a potential lactic acid bacterium which can produce large amount of lactic acid and has the ability to release chitin (demineralization) from shrimp-shell-waste. Among 11 bacteria tested, strain 15 was the strongest lactic acid producer yielding 1.09% (v/v) lactic acid in the media with a pH of 4.15 after 6 days incubation at 37 °C. After that, strains 17 and 23 produced 0.79 and 0.74% of lactic acid respectively. These three strains were selected for further experiments on various kinds of media using two-day incubation periods. No strains produced lactic acid well in MRS media containing lactose. The best medium for lactic acid production by strains 15 and 17 was MRS containing glucose, molasses or mixture of molasses and shrimp shell waste. Fermentation of shrimp shell waste using strain 15 caused an increase of viscosity reflecting an increase of soluble chitin in the media.

Key words: chitin, lactic acid bacteria, recovery processing, lactic acid

Recent interest in chitin and chitosan in industrial use is reflected by an increasing number of meetings in Europe, Asia and America, and papers published on chitin-chitosan solubilization (George *et al*. 1999). The potential use of chitin and chitosan is widely recognised, and many new applications have been developed. Currently, chitin, chitosan and their derivatives are widely used in chemical, medical, pharmaceutical, cosmetics, food technology and water treatment industries. However, the use is restricted to specific applications because of the high cost of technical-grade chitin and chitosan. Greater industrial use of chitin will be possible, if its manufacturing processes were made cheaper. Chitin and the by-products e.g. carotene-protein, could be recovered from crustacean waste products.

Marine by-products rich in chitin and protein are renewable resources present in large amounts in many countries. Shrimp production in Indonesia is around 240,000 metric tons per year (BRKP 2005), and non-edible wastes such as carapace and exoskeleton can make up to 50-60% of this volume. Due to their high availability and their chemical composition (27% chitin, 40% protein, and 35% minerals), shrimp residues are prime candidates for use as a raw material for producing chitin-chitosan. Shrimp shell waste is partially used as animal feed but most of it is discarded causing serious ecological problems. It has also been used as an ingredient in culture media; for extraction of pigments and proteins to formulate animal feed; for reducing nematode populations in soils; for producing single-cell protein, and for enzyme immobilization.

The traditional methods for commercial preparation of chitin from crustacean shell waste have remained essentially unchanged since first proposed (Rigby 1934). The traditional processes are mechanical grinding, demineralization with 1 N hydrochloric acid and deproteinization with 3-5% sodium hydroxide at 90-100 °C. These chemical treatment procedures potentially pollute the environment and the resulting wastes have to be treated before being discarded. The treatment has been identified as the most important factor contributing to the high price for chitin and chitosan products (Muzzarelli 1990). Thus, in order to increase chitin use and diminish the emission of pollutants, a less expensive and more environmentally friendly method for large scale extraction of chitin needs to be developed.

Lactic acid fermentation combined with chemical treatment has been studied as an alternative method of chitin recovery that reduces the amount of alkali and acid needed. The removal of protein and calcium from shells is by a combination of enzymatic activity and mineral solubilization by organic acid produced in bacteria growth (Shirai *et al*. 1997; Zakaria *et al*. 1998; Hsu and Wu 2002; Luis *et al*. 2003).

The purpose of this research was to identify a potential lactic acid bacterium which could produce large amounts of lactic acid for demineralization in the shrimp-shell-waste process.

MATERIALS AND METHODS

Materials. Bacterial culture media including deMan Rogosa and Sharpe (MRS), MRS Broth, MRS Agar and ingredients including yeast extract, peptone, and glucose were purchased from Merck KGaA, Darmstadt, Germany. Lactobacilli Broth AOAC was purchased from Difco Laboratories, USA. Molasses was obtained from Pakistan.

Microorganisms and Growth Conditions. The lactic acid bacteria tested were six strains (3, 4, S5, 13, 15, and 17) isolated from Indonesian traditional food (purchased from BPPT Culture Collection) and five strains (5, 9, 23, 95, and 135) isolated from tempe (fermented soybean cake) purchased from the Food Microbiology Laboratory, Hamburg University. The lactic acid bacteria were maintained on MRS broth containing 30% v/v glycerol as cryoprotectant and kept at -80 °C. MRS agar slopes were prepared and stored at 4 °C.

The inoculum was prepared using MRS broth with a loopful of cells from a slope of MRS agar and incubated at 37 °C for 24 hours.

Shrimp Shell Fermentation. Screening the best lactic acid bacteria for demineralization of the shrimp shell was carried out on MRS medium at pH 7.0 which contained 6% of shrimp shell and molasses instead of glucose. An inoculum of 70 ìl of each cell suspension was subcultured into 7.0 ml medium. The culture was incubated on a shaking water bath (Gyrotory Shaking Water bath model G 76, New Brunswick Scientific, USA) at 37 °C for six days. Samples for determination of pH and lactic acid production were collected every 24 hours.

In further experiments the lactic acid production of the three selected bacteria was observed on the MRS media containing molasses, lactose, or shrimp shell waste, incorporated with either meat extract or peptone or K_2HPO_4 or $MgSO_4$.

Analytical Procedures. The moisture content of shrimp shell waste was measured by oven drying samples at 105 °C for 90 min. Ash content was determined by burning samples in a crucible at 800 °C in a muffle furnace for 90 min. Mineral content was measured by titration with Titrisol (Titriplex solution B, Merck). Protein content was calculated using the following equation:

% Protein = 1 - (% Ash without NaOH treatment - % Ash with NaOH treatment)

Viscosities are determined using a suspended level Ubbelohde viscometer equipped with three bulbs situated at different heights above the bottom of the capillary. The system DMAc (N,N-dimethylacetamide)/LiCl (lithium chloride) is prepared by weighing rapidly the salt and adding the solvent required. Chitin solutions in 5% (w/w) LiCl/DMAc were prepared as follows; a dry chitin sample (5 mg) was suspended in 20 ml of 5% LiCl/DMAc, and then the mixture was stored at room temperature with occasional shaking until complete dissolution was achieved.

The pH of samples and fermentation broth was measured using a pH electrode (pH-mV-meter, Knick). Lactic acid concentrations were determined by HPLC (Hitachi model D-2500) after heating at 80 °C for 10 min and centrifugation at 13,000 x g at room temperature for 10 min. Five µl aliquots of the filtrate were injected onto a liquid chromatography using a Ion Gard ORH 801 column and eluted with 180 µl H_2SO_4 in 1.0 l milliQ water. Operationing conditions were 0.6 ml min^{-1} flow rate and 60 °C. The chromatograph was fitted with a RI detector (LaChrom RI Detector L-7490, Merck). A Fourier Transforming Infra-Red (FT-IR) spectrometer was employed to confirm the structure of chitin.

RESULTS

Shrimp Shell Waste. Ash and chitin contents in shrimp shell waste from Indonesia were 21.67 and 26.13% respectively. The results indicate that they were lower than the minced waste from cold water areas (George *et al*. 1999). Table 1 shows the characteristics of shrimp shell waste obtained from the shrimp fishing industry in Lampung, Indonesia.

Screening of Lactic Acid Bacteria (LAB). The eleven strains tested produced varying amounts of lactic acid. After 6 days fermentation at 37 °C, strain 15 was identified as the strongest lactic acid producer with a pH 4.15 and it yielded 1.09% (v/v) lactic acid in the medium (Table 2), while strains 17 and 23 produced 0.79 and 0.74% (v/v) lactic acid, respectively. These three strains were selected for further experiments.

The Changes of pH during Fermentation Process. In the course of the fermentation the pH gradually decreased from 7.0 to 4.7 in the media containing shrimp shell and molasses. Results were not in agreement with those observed in MRS broth media containing glucose that the pH decreased from 5.5 to 3.5 after three days incubation. However, in all cases the decrease of pH reflected the amount of lactic acid production.

Lactic Acid Production in Various Media. Three strains were tested in various kinds of media for two days incubation at 37 °C (Table 3). No strain produced well lactic acid in the lactose media (media H). The best medium for growing strains 15 and 17 is MRS broth, which is a selective medium for producing lactic acid. Strain 15 was also the best lactic acid producer when grown on medium containing molasses and

Table 1 Characteristics of the shrimp shell waste from Indonesia

Variable	% w/w
Moisture	28.21
Ash	21.67
Protein	37.84
Mineral	36.03
Chitin	26.13

Table 2 Lactic acid production in MRS broth media* at 37 °C for 6 days incubation time as a function of bacterial strain

Strain	Final pH	Lactic acid (% v/v)
3	5.20	0.30
4	5.00	0.35
S 5	5.20	0.19
5	5.55	0.16
9	5.40	0.23
13	> 8.00	0.07
15	4.15	1.09
17	4.45	0.79
23	4.60	0.74
95	4.80	0.57
135	4.85	0.51

*Glucose was replaced by molasses

Table 3 Production of lactic acid on various media by 3 bacterial strains for 2 days

Strain	% (v/v) Lactic acid in media							
	A	B	C	D	E	F	G	H
15	1.41	1.22	0.70	0.69	0.47	0.33	0.22	0.05
17	1.42	0.59	0.75	0.72	0.08	0.33	0.20	0.06
23	0.25	0.76	0.37	0.20	0.17	0.10	0.06	0.03

A: media is MRS broth; B: media is MRS broth but replacing glucose by molasses; C: media is molasses (50 g l^{-1}) and meat extract (5 g l^{-1}); D: media is molasses (50 g l^{-1}) and peptone (10 g l^{-1}); E: media is molasses (50 g l^{-1}), K_2HPO_4 (2 g l^{-1}), and $MgSO_4$ (0.2 g l^{-1}); F: media is molasses (50 g l^{-1}); G: media is molasses (50 g l^{-1}) and shrimp shell waste (160 g l^{-1}); H: media is lactose (20 g l^{-1})

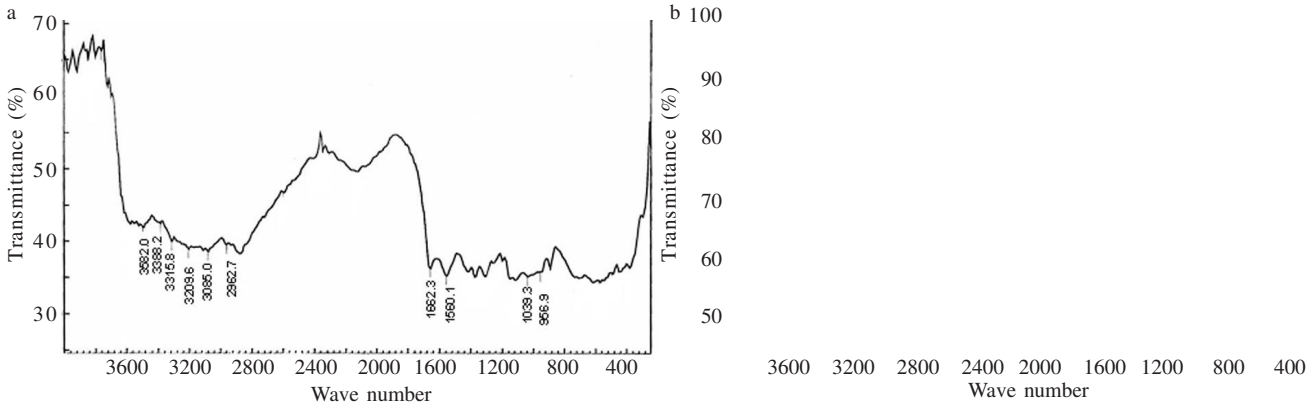

Figure 1 FT-IR of commercial chitin (a) and chitin from lactic acid bacteria fermentation strain no. 15 on shrimp shell waste (b).

shrimp shell waste, reflecting the ability of the strain to digest the shrimp shell waste.

Fermentation of Shrimp Shell Waste. Results from FT-IR spectrometer for fermented shrimp shell waste with strain 15 show that peaks of O-H; N-H; CH_3–CH_2; ketone and 1,4 glucosamine were at 3496.1 cm^{-1}; 3272.4–3218.8 cm^{-1}; 2954.9 cm^{-1} ; 1659.2–1543.8 cm^{-1} and 1074.6 cm^{-1}, respectively (Figure 1b). Those results were similar with those observed in commercial chitin (Figure 1a).

DISCUSSION

Lactic acid fermentation is one of the methods currently used in the biological procedures for the demineralization of chitin. It purifies chitin by hydrolysis of the protein, which are bonded to the chitin in the shell waste (Zakaria *et al*. 1998; George *et al*. 1999). During the fermentation, based on HPLC analysis strain 15 had the ability to produce well lactic acid from glucose than the medium containing lactose. This result confirmed that strain 15 is a lactic acid bacterium.

Fermentation of shrimp shell waste using strain 15 resulted to increase the viscosity up to 60 times compare that of unfermented one. The solubility of purified chitin from protein of shrimp shell waste increased the viscosity in the fermentation media. Strain 15 is a lactic acid bacterium producing lactic acid in the glucose media. The increase of viscosity in the fermentation of shrimp shell waste show that strain 15 may be a good candidate for demineralization chitin from shrimp shell waste (George 1999). Further work is needed in order to establish whether a process based on lactic acid fermentation could be good quality and economically feasible.

ACKNOWLEDGEMENT

I am grateful to the DAAD for financial supporting through a DAAD Scholarship granted to the author through a Special programme for Biosciences 2003. The author would like to thank Bernward Bisping, University of Hamburg for assistance, providing the laboratory facilities and kindness. Finally, I would like to Gerd Mueller von der Haegen and to acknowledge the assistance and support of all the SeeLab and Food Microbiology Laboratory members.

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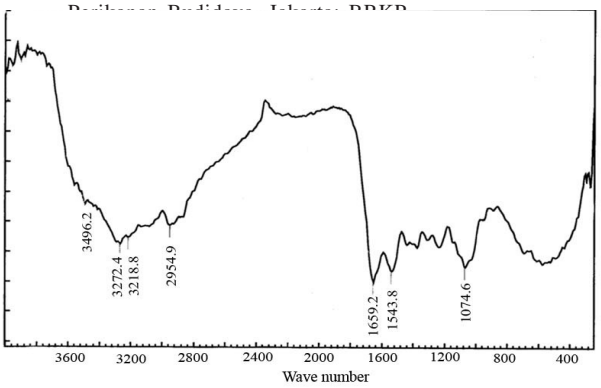

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