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Full Length Research Paper

Melanoidin decolorization mechanism and some properties of *Issatchenkia orientalis* No.SF9-246

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***Issatchenkia orientalis* No. SF9-246 cell showed both melanoidin (MP) adsorption and degradation abilities and its MP-adsorption yield was reduced by acclimatization with medium containing MP. The living cell showed the MP-adsorption ability of 15%; higher than the autoclaved cell (dead cell). The MP-adsorption ability of the deteriorated cell (MP-adsorbed cell) was recovered by washing with 0.1 mol L^{-1} H_2SO_4 solution. Un-expectantly, the MP-adsorption capacity of the deteriorated cell was about 100 to 150% increased after washing with 0.1 mol L^{-1} H_2SO_4 solution. Also, the strain showed the highest biomass production yield of 43.6±2.5 g L^{-1} in the medium containing MP within 7 days cultivation and the crude protein content of the biomass was 36.38±1.12%. In addition, the strain produced intracellular glutathione *S*-transferase enzymes types Y-1 and Y-2 (GST Y-1 and GST Y-2) and GST Y-1 production was induced by MP.**

Key words: Adsorption, decolorization, *Issatchenkia orientalis*, melanoidin, molasses wastewater.

INTRODUCTION

Ethanol production industry from molasses generated a large volume wastewater that contained high organic matters and colored substances (Monica et al., 2004). Melanoidin pigment (MP) (dark brown pigment) is the main colored substances of molasses wastewater (Monica et al., 2004). This colored substance was hardly biodegraded in both conventional aerobic and anaerobic wastewater treatment systems, such as activated sludge system (AS) and methane fermentation system, respectively (Metcalf and Eddy, 2004; Sirianuntapiboon, 1999). However, the entire wastewater should be treated before discharging it into the environment where the chemical treatment process was usually applied (Metcalf and Eddy, 2004), but the treatment cost was quite high and the large amount of chemical waste was generated (Metcalf and Eddy, 2004; Sirianuntapiboon et al., 2004a, b). According to the stated results, the development of the new wastewater treatment system with low operating cost, easy operation, high efficiency and stable removal yield was expected.

The biological treatment process was one of the promising and interesting methods to be investigated (Bromley-Challenor et al., 2000; Raghukumar and Rivonkar, 2001; Sirianuntapiboon et al., 2004a, b). Many researchers tried to isolate and apply the special microorganism having MP removal ability into the conventional biological wastewater treatment system, which resulted to increase in the MP removal yield (Raghukumar and Rivonkar, 2001). The MP removal ability was found in several kinds of microorganisms such as mushroom, fungi (Sirianuntapiboon et al., 1995; Kim and Shoda, 1999; Dahiya et al., 2001; Raghukumar and Rivonkar, 2001), bacteria (Sirianuntapiboon et al., 2004a)

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Abbreviations: AS, Activated sludge system; **BOD_5,** biochemical oxygen demand; **COD,** chemical oxygen demand; **GST,** glutathioneS-transferase; **MP,** melanoidin pigment; **MWW,** molasses wastewater, stillage; **MYGPA,** malt extract-yeast extract-glucose-peptone agar; **MYGPA-MP,** malt extract-yeast extract-glucose-peptone agar containing MP; **MYGPB,** malt extract-yeast extract-glucose-peptone broth; **MYGPB-MP,** malt extract-yeast extract-glucose-peptone broth containing MP; **PCR,** polymerase chain reaction; **SDS,** sodium dodecyl sulfated; **SCP,** single cell protein; **SWW,** synthetic wastewater; **SWW-MP,** synthetic wastewater containing MP; **TEM,** transmission electron-microscope; **U-MWW,** untreated molasses wastewater.

and yeast (Sirianuntapiboon et al., 2004b; Tondee et al., 2008). However, the application of those strains into the biological wastewater treatment system had many problems, especially, instability of MP removal yield and microbial population. The yeast strain, *Issatchenkia orientalis* No.SF9-246 was isolated from the previous work (Tondee et al., 2008) and showed the highest MP removal yield of 91.24% with raw molasses wastewater (stillage). This strain was most suitable to be applied for treatment of molasses wastewater according to its high removal abilities on both organic matters and MP (Tondee et al., 2008). But the MP decolorization mechanism of this strain was still unclear. In this study, MP removal mechanism of *I. orientalis* No.SF9-246 was observed. The application of *I. orientalis* No.SF9-246 cell as the MP-adsorbent was tested. Also, some properties of the strain were investigated such as biomass protein content and metabolites.

MATERIALS AND METHODS

Microorganism

Yeast strain, *I. orientalis* No.SF9-246, having high MP decolorizing activity from the pervious work (Tondee et al., 2008) was used in this study. The strain was cultivated and maintained on malt extract-yeast extract-glucose-peptone (MYGP) agar: MYGPA (Difco Laboratories, 1967).

Preparation of melanoidin (MP) solution

MP solution was prepared from the stillage (raw molasses wastewater) of Sang-Som alcohol distillation factory, Nakhon Pathom, Thailand (Table 1). The stillage was centrifuged at 6,000 × g for 15 min. The supernatant was evaporated five times of the original concentration in a low temperature vacuum evaporator (Potavapor Buchii Model RE 120, Japan) at 50°C. The concentrated solution was dialyzed (dialyzing tube type: molecular weight cut off 10,000 Dalton) against running tap water for 2 days and then against de-ionized water for 2 days. The non-dialyzable MP solution thus prepared was used for this experiment as MP solution after dilution with distilled water to a concentration corresponding to an optical density of 3.5 units at a wavelength of 475 nm.

Medium for acclimatization of *I. orientalis* No.SF9-246 for MP adsorption test

Two types of liquid medium used to acclimatize *I. orientalis* No.SF9-246 before testing MP-adsorption capacity, were malt extract-yeast extract-glucose-peptone broth: MYGPB (Difco Laboratory, 1967) and MYGPB containing MP: MYGPB-MP. The composition of MYGPB-MP was similar to that of MYGPB except for distilled water. MP solution was used instead of distilled water. For the solid media, MYGPA-MP 2% agar was added into the MYGPB-MP.

Preparation of *I. orientalis* No.SF9-246 cell for MP adsorption test

Two types of *I. orientalis* No.SF9-246 cell as types A and B were prepared by cultivating the strain in MPGPB and MPGPB-MP for 3 days each, respectively. Both types of yeast cell were used as resting cell (living cell) after washing with 0.1 mol l^{-1} phosphate buffer pH 7.0 (resting cell type A and resting cell type B) and autoclaved cell (dead cell) after being autoclaved at 110°C for 10 min for 2 times (autoclaved cell type A and autoclaved cell type B).

MP adsorption stability test

The MP-adsorption capacity of *I. orientalis* No.SF9-246 cell was tested with MP solution in jar test reactor (Sirianuntapiboon and Saengow, 2004) under constant temperature of 30°C for 3 h. The reaction mixer contained *I. orientalis* No.SF9-246 cells at the various concentrations of 1,000, 1,500, 3,000, 3,500 and 4,000 mg L^{-1}. The MP-adsorption capacity was analyzed by using Freundlich's adsorption isotherm equation according to the multilayer adsorption mechanism of bio-sludge (Sirianuntapiboon and Saengow, 2004).

Elution of MP from MP-adsorbed cell

The MP-adsorbed cell was prepared by incubation *I. orientalis* No.SF9-246 cell in MP solution at 30°C in jar test reactor for 3 h. Then, the MP-adsorbed cell was washed with various kinds of eluting solution as distilled water, 0.1 mol l^{-1} phosphate buffer pH 7.0, 0.1% Tween 80, 0.1% triton X 100, 0.1% cholic acid, 0.1 mol l^{-1} H_2SO_4, 0.1 mol l^{-1} NaOH, and 0.1% SDS solutions. The reaction mixture was centrifuged at 6,000×g for 10 min. The eluted-MP in the supernatant was determined by spectrophotometer under a wavelength of 475 nm (Sirianuntapiboon et al., 1995).

Growth of *I. orientalis* No.SF9-246 cell for transmission electron microscopic determination

10 ml of *I. orientalis* No.SF9-246 cell suspension (9.0×10^{12} cells/ml) was inoculated into MYGPB in a 500-Erlenmeyer flask and incubated at 30°C on a reciprocal shaker (125 oscillations min^{-1}, 7 cm stroke) for 3 days. Then, the cultivated cell was harvested and washed with 0.1 mol l^{-1} phosphate buffer (pH 6) for 3 times. The washed cell was re-cultivated for 3 more days in MYGPB-MP. After that, the MPGPB-MP cultivated cell was washed with 0.1 mol l^{-1} phosphate buffer (pH 6) for 3 times and transferred back into MYGPB and cultivated for 3 more days. All the experiments were carried out under aseptic condition. The cultivated cells from each step was collected and 3 times-washed with 0.1 mol l^{-1} phosphate buffer (pH 6) before morphological observation by light microscope and transmission electron microscope (TEM).

Sample preparation for transmission electron microscope (TEM)

I. orientalis No.SF9-246 cell, collected from each step of cultivation was prefixed in 4% para-formaldehyde for 2 h and followed by 1% osmium tetra-oxide in 0.1 mol l^{-1} phosphate buffer (pH 7.3) for 2 h. And then, it was strained with 2% aqueous uranyl acetate for 1 h. The strained cell was washed thoroughly with distilled water and dehydrated by sequential soaking in 50, 70, 80, 90 and 100% ethanol for 30 min each. The treatment with 100% ethanol was repeated for three times. The dehydrated cell was infiltrated with and embedded in spur's resin and then baked at 70°C for 10 h. An ultra-microtome (Ultratome V; LKB, Sweden) was then used to cut 60 to 90 nm sections. The stated section was stained with lead citrate and uranyl acetate before examination by a transmission electron microscope (JEM 200 CX; JEOL) at 80 kV (Griffin et al., 1990; Bozzola and Russell, 1992).

Table 1. Chemical composition of *I. orientalis* No.SF9-246 cell and the other fugal strains.

Chemical properties	Raw molasse wastewater (stillage) U-MWW[a]
pH	4.6-5.5
Temperature (°C)	78-84
Color intensity (475 nm)	38±5
Reducing sugar (mg ml^{-1})	207.8±4.0
COD (mg l^{-1})	105,851±3,575
BOD_5 (mg l^{-1})	22,985±795
TKN as N (mg l^{-1})	2,326±80
Ammonuim: NH_4^+ as N (mg l^{-1})	120.5±15.9

[a]:U-MWW is the stillage from an alcohol factory collected from wastewater storage pond of Sang-Som factory, Nakornpathom province, Thailand.

Examination of MP accumulation in *I. orientalis* No.SF9-246 cell

The detection of accumulated-MP on the cell components (cell wall, endoplasmic reticulum, Golgi body and cell membrane and cytoplasm) of *I. orientalis* No.SF9-246 cell was studied. The strain was cultivated in MYGPB-MP at 30°C for 2 days (late log phase state). The cultivated cell (optical density at 660 nm of the culture: OD_{660nm} was 1 unit) was harvested by centrifugation at 6,000 × g for 5 min and then, washed with 10 ml sterile distilled water. The washed-cell was re-suspended with 1 ml of 0.1 mol L^{-1} Tris-HCl (pH 9.4) and 10 µl of 1 mol L^{-1} dithiotheritoal (DTT). The mixture was incubated at 30°C for 10 min and then 1 ml spheroplast medium was added. After that, the 10 µl of suspension was added with 1 ml sterilized water. The optical density of the mixture was determined (OD_{660nm} = a). 0.25 mg of zymolyase was added into the mixture. The 10 µl of the mixture was suspended with 1 ml of sterilized-distilled water. The optical density of the stated mixture was re-checked to compare with the previous observation (OD_{660nm} must be less than a/10). And then, the mixture (spheroplast cell suspension) was kept on ice (the spheroplast cell shape could be observed under light microscope). The stated spheroplast cell suspension was used to extract the cell components by the cell component extraction protocol as follows: Step 1: 1 ml mixture (cell suspension) was suspended in 15 ml sterilized-tube and 1.25 ml of 1.4 mol L^{-1} sorbitol was added. After that, the cells was harvested by centrifugation at 6,000 ×g for 5 min and re-suspended in lysis buffer (mixture of 1 mM of DTT, 20 µg ml^{-1} of PMSF and 100 µM of 20 mg ml^{-1}). The mixture was centrifuged at 6,000 ×g for 5 min to harvest the precipitate; cell wall sample (precipitate: ppt.). Step 2: 0.8 ml supernatant from step 1 was added with 0.8 ml lysis buffer and 0.1 ml cussion solution (80% sucrose in 20 mM HEPES KOH solution pH 6.8), then it was centrifuged at 13,000 ×g for 15 min. The precipitate was collected as endoplasmic reticulum sample (P13). Step 3: 0.5 ml of supernatant from step 2 was added with 0.8 ml of lysis buffer and 0.1 ml of cussion solution and then was centrifuged at 100,000 × g for 1 h. The precipitate was collected as cell membrane sample (P100) and cytoplasm (S100). The four samples mentioned earlier; ppt, P13, P100 and S100 were suspended with lysis buffer to the final volume of 100 µl, then, 25 µl of 50% tri-chloroacetic acid (TCA) was added and the mixers were kept on ice for 1 h. The mixtures were centrifuged at 13,000 × g for 10 min. The precipitates were added with 100 µl of acetone (-20°C) and then it was centrifuged at 13,000 ×g for 10 min. The precipitate was re-suspended with 9 µl of 2 X SDS- polyacrylamide gels (PAGE) buffers and 1 µl of βME and mixed by vertex. The mixtures were incubated at 95°C for 5 min and were kept on ice. The samples were analyzed by thin layer chromatography technique on SDS polyacrylamide gels (PAGE) in 10 xTris/Glycine/SDS buffer for SDS PAGE application at 24 mA for 90 min.

Extraction and determination of MP decolorization enzyme of *I. orientalis* No.SF9-246

The strain was cultivated in 500 ml of shaking flask containing 100 ml of MYGPB and MYGPB-MP at 30°C for 3 days. Both cultivated-cells were harvested by centrifugation at 6,000 ×g for 15 min at 4°C. Then, they were re-suspended with 1.5 ml extraction buffer (the extract buffer was the mixture of 50 mM potassium phosphate buffer (pH7), 1 mM EDTA, 10 mM sodium sulfite and protease inhibitors). Glass beads were added in the mixture. The mixture was mixed by vortex for 1 min and kept on ice for 1 min. The stated process was repeated for 10 times. The supernatants of the reaction mixtures were harvested by centrifugation at 6,000 × g, at 4°C for 15 min and filtered (mesh size of the filter is 0.22 µm). The filtrates were used as the cell free extract (crude intracellular enzyme) suspensions to determine the MP-decolorization activity. To determine the MP-decolorizing activity of the cell free extract suspension, the filter paper disc (0.5 cm in diameter) was immersed into the cell free extract suspension and placed on the agar plate containing MYGPA-MP. The clear zone around the filter disc was observed and measured everyday during 3 days incubation at 30°C.

Expression and purification of glutathione S-transferase (GST) gene

Yeast-RNA extraction

I. orientalis No.SF9-246 was cultivated in MYGPB-MP at 30°C on the reciprocal shaker (125 oscillation min^{-1}, 7-cm stroke) for 1 day. The cultivated cell was harvested by centrifugation at 6,000 × g for 10 min. The harvested cell was used for isolation of the yeast-RNA by RNeasy mini kits (QIAGEN, Japan). The cell free extract sample was collected as crude yeast-RNA sample (the optical density of the sample was determined by spectrophotometer at a wavelength of 260 nm).

Precipitation of extracted RNA

All purification steps were carried out at 4°C. One volume of the cell free extract from the earlier section was added with 0.1 volume of 3 mol l^{-1} sterilized-sodium acetate pH 5.3 and 3 volume of absolute ethanol. After mixing well by vortex mixer, the mixture was kept at -

80°C for 15 min. The precipitate was harvested by centrifugation at 8,000 × g for 15 min. The precipitate was washed by 3 volume of 70% ethanol. The precipitate was collected by centrifugation and suspended in RNA free water for the yeast-RNA sample.

m-RNA selective PCR

The coding region of glutathione S- transferase enzyme types Y-1 and Y-2 genes (GST Y- 1 and GST Y-2 genes) were isolated by PCR amplification using the yeast RNA sample prepared from the previous section as a template for RNA polymerization with RNA polymerase. Yeast mRNA gene was amplified with 3' GST Y-1 oligonucleotide (GCGAAGAAGTTGCTAAACATGAACTTTTGG-3') and 5'GST Y-1 oligonucleotide (5'CGGTACCCTCTACATT CTTCCACCATCCCC) for GST Y-1 and 3' GST Y-2 oligonucleotide (GGTGAGGTCTCCCAGCATCCAATTATCAAG AA-3') and 5' GST Y-2 oligonucleotide (5'- TGTTTATATCAAGCCACACACCCCA GAGG) for GST Y-2. The PCR mixture contained 25 µl 2X mRNA selective PCR buffer (Takara), 10 µl $MgCl_2$, 5 µl dNTP/analog mix, 1 µl RNase inhibitor, 1 µl 20 µM 5' and 3' primer GST Y-1 (or GST Y-2), 1 µl of 1 µg yeast total RNA and 4 µl RNase free water. 1 µl AMV reverse transcriptase XL and 1 µl AMV-optimized Taq polymerase were added to 48 µl PCR mixture before running the PCR process. The operation condition of the PCR process was as followed: RNA polymerization step at 42°C for 15 to 30 min, denaturation at 85°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min and the final extension step at 72°C for 7 min. The DNA-PCR products were collected at 15, 20 and 25 cycles. The DNA amplified product was analyzed by electrophoresis on sterile 1% agarose gels in sterile 1X TBE buffer (0.045 M Tris-borate, 0.001 M EDTA pH 8). Gels were stained with ethidium bromide, visualized and photographed under UV light. Fragment sizes were estimated by comparison against a DNA Marker (1-10 kbp; Takara, Japan). The results of 550 and 555 bp DNA fragments were represented as GST Y-1 and GST Y-2 genes, respectively.

Analysis of yeast RNA by northern hybridization

All operations were carried out under aseptic technique to avoid the outsourcing contamination. The mRNA selective PCR products, GST Y-1 and GST Y-2 genes were labeled by DIG high prime DNA labeling and detection starter kit II (Roche Applied Science, Germany) according to the manufacturer's instruction with a slight modification.

Determination of chemical composition of *I. orientalis* No.SF9-246 cell

I. orientalis No.SF9-246 was cultivated with synthetic media (SWW) containing with and without MP under aerobic condition using batch type system for 1, 3 and 7 days. SWW contained 2.5% glucose, 0.1% NH_4Cl, 0.1% KH_2PO_4 and 0.05% $MgSO_4$ in 100 ml distilled water and the pH was adjusted to 5. The chemical composition of SWW-MP was similar to that of SWW, excepted for the distilled water. MP solution (OD_{475nm} of 3.5 units) was used instead of distilled water. The chemical composition of the cultivated cell as dry matter, total nitrogen, TKN, crude protein, lipid and total ash were determined by using the standard method of AOAC (1980). Crude protein content was calculated by the equation of 6.25 X total Kjeldahl nitrogen (TKN).

Assay

MP-decolorization activity was measured as the decrease in color intensity of the medium. The color intensity of the sample after dilution with 0.1 acetate buffer (pH 5.0) was measured with a spectronic 21 system (Bausch and Lomb, Sweden) at a wavelength of 475 nm. The MP-decolorization yield was taken as the extent of the decrease in absorbance at 475 nm relative to the initial absorbance (Sirinuntapiboon et al, 1995). Dry weight of cell mass was measured after drying at 105°C for 24 h. Chemical oxygen demand (COD) and biological oxygen demand (BOD_5) were measured by a standard method for examination of water and wastewater (APHA, AWWA, WPCF, 1998). Reducing sugar was measured by the method of Somogyi-Nelson (Nelson, 1944; Somogyi, 1952).

Statistical analysis method

Each experiment was repeated at least 3 times. All the data were subjected to two-way analysis of variance (ANOVA) using SAS windows version 6.12 (SAS Institute, 1996). Statistical significance was tested using the least significant difference (LSD) at the $p < 0.05$ level and the results are shown as the mean ± standard deviation (X ± SD).

RESULTS

Observation of the MP-decolorization activity of cell free extracts of *I. orientalis* No.SF9-246 culture

The experiment was carried out by using agar plate technique. The giant clear zone (≥1 cm in diameter) was detected around the filter disc containing cell free extract prepared from cell suspension cultivated in MYGPB-MP while the small clear zone (less than 0.6 cm) was detected in the case of MYGPB (data not show).

Observation of MP accumulation in *I. orientalis* No.SF9-246 cell by thin layer chromatography

MP was detected on the cell wall sample of the 2 days-culture, while it could not be detected on the cell membrane, endoplasmic reticulum and Golgi body samples. It could be suggested that, MP was accumulated on the cell wall section of the 2 days-cultured cell.

Electron microscopic study of *I. orientalis* No.SF9-246 cell grown in MYGPB and MYPGB-MP

I. orientalis No.SF9-246 cell was harvested from MYGPB-MP and was dark-brown color even when it was washed with 0.1 mol L^{-1} phosphate buffer (pH 6) for several times (more than 3 times) while the MYGPB cultivated-cell was colorless. Cross-section of the cells that were acclimatized with MYGPB-MP and MYGPB are shown in Figures 1a and b, respectively. After 3 days cultivation in MYGPB, a lot of electron dense materials were observed in the cytoplasm and around the cell membrane. However, some of the electron dense materials had disappeared after 2 weeks-cultivation. In contrast, the cell

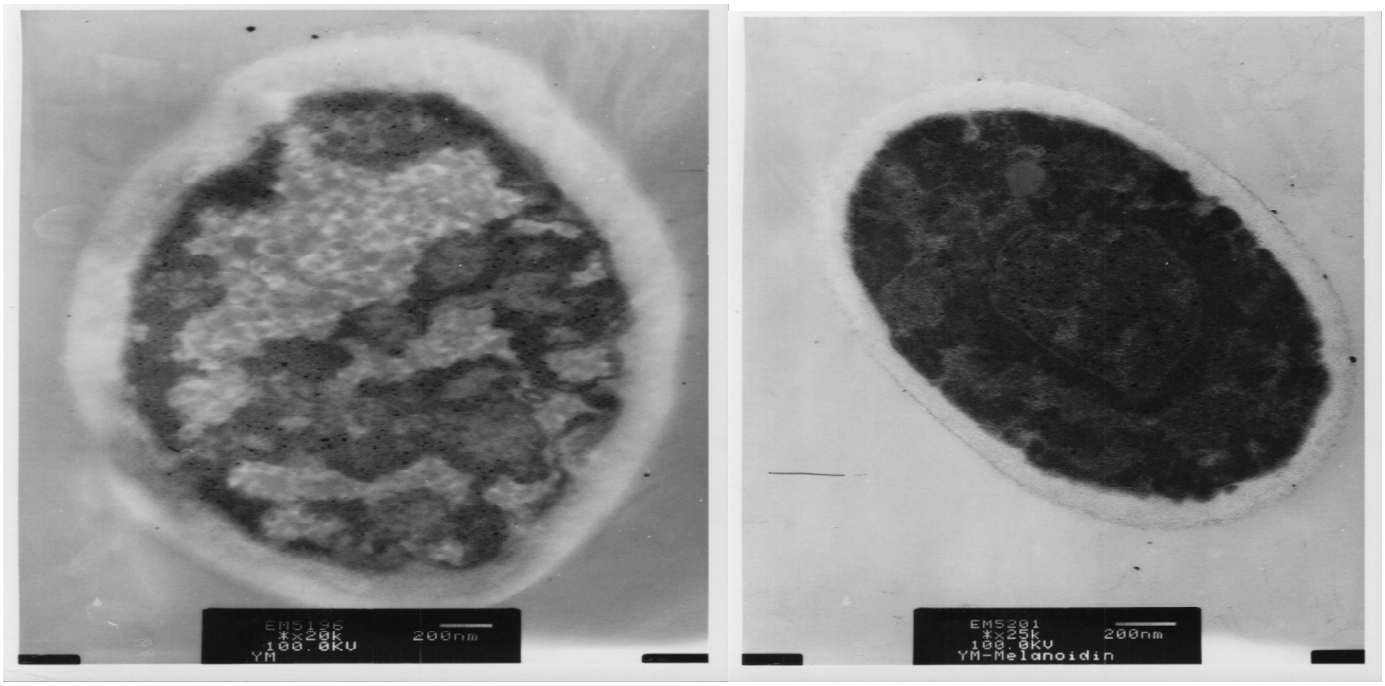

Figure 1. Electron micrographs of *I. orientalis* No. SF9-246 in MYGP with and without MP. (A), Cross-section of 7 days-cultivated cell, grown in MYGP medium without MP, showing well-defined cell organelles such as cell wall and cell membrane; (B), cross-section of 7 days-cultivated cell, grown in MYGP medium with MP, showing electron dense materials distributed in the cytoplasm.

harvested from MYGPB did not have any electron dense materials (Figure 1b).

Comparative electrons microscopic study of *I. orientalis* No.SF9-246 cells was cultivated in various types of media. This was done by collecting the cell at each step of the cultivation and the results are shown in Figure 2. The cell cultivated on MYGPB (Figure 2a) did not have any spots of electron dense material in its cytoplasm. After transfer to MYGPB-MP and cultivation for 3 more days, the cell (Figure 2b) changed intracellular and copious electron-dense material appeared in the cytoplasm and around the cell membrane. However, these materials disappeared when the cell was transferred back to the MYGPB and cultivated for 3 more days, although, the color of the culture filtrate did not change during this final stage of cultivation.

Adsorption of MP by *I. orientalis* No.SF9-246 cell

Both autoclaved and resting cells type A showed the MP-adsorption yield of about 15% higher than that of both autoclaved and resting cells type B as shown in Table 2. The resting cell type A showed the highest MP-adsorption yield of 27.8±1.3%. However, its MP-adsorption yield was about 10 to 15% reduced by autoclaving.

Elution of adsorbed-MP from *I. orientalis* No.SF9-246 cell

The adsorbed-MP was easily eluted from both the autoclaved and resting cells types A and B by washing with 0.1% SDS and 0.1 mol/L H_2SO_4 solutions, but it hardly was eluted by washing with 0.1% cholic acid solution and distilled water (Table 3). Also, the adsorbed-MP was easily eluted from the MP-adsorbed cell that was acclimatized with MYGPB than that with MYGPB-MP. However, 0.1 mol L^{-1} H_2SO_4 and 0.1% SDS solutions were more suitable to be used as the eluent according to the highest elution yield of more than 85% in all types of *I. orientalis* No.SF9-246 cell.

For further investigation, the stability of MP-adsorption capacity after washing with 0.1 mol L^{-1} NaOH, 0.1 mol L^{-1} H_2SO_4, and 0.1 mol L^{-1} phosphate buffer at pH 6 or 0.1% SDS solutions was determined.

Stability of MP-adsorption capacity

The deteriorated (MP-adsorbed) cells types A and B of *I. orientalis No.SF9-246* (both autoclaved and resting cells) could be reused for MP-adsorption after washing with0.1 mol l^{-1} H_2SO_4 solution. Un-expectantly, the MP

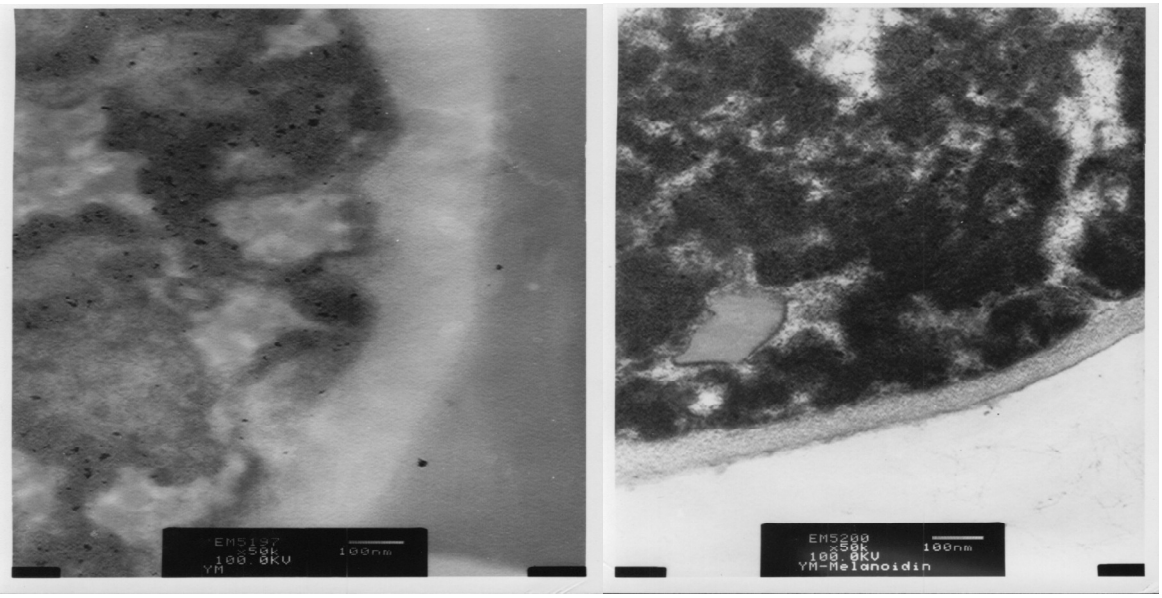

Figure 2. Electron Micrographs of *I. orientalis* No. SF9-246 collected at various stages of cultivation. (A), Cross-section of *I. orientalis* No. SF9-246 cell that had been grown in MYGP for 7 days (100,000X magnification), showing the clear cytoplasm and the cell membrane; (B), Cross-section of *I. orientalis* No. SF9-246 cell that had been grown in MYGP for 7 days and then in MYPG-MP for another 4 days (100,000X magnification), showing electron-dense materials distributed in the cytoplasm and cell membrane.

Table 2. MP-adsorption yields of *I. orientalis* No.SF9-246 under various conditions.

Acclimatization media	Type of cell	Adsorption yield (%)	Relative activity (%)
MYGPB[a]	Resting cell type A	27.8 ± 1.3	100.0
	Autoclaved cell type A	25.3 ± 0.8	91.0
MYGPB-MP[b]	Resting cell type B	24.5± 0.5	87.2
	Autoclaved cell type B	20.6 ± 0.8	74.1

[a], MYGPB; Malt extract-yeast extract-glucose-peptone broth (Difco Laboratories, 1967); [b], MPGPB-MP; MYGPB (Difco Laboratories, 1967) containing MP.

adsorption capacity of the deteriorated cell (1st MP-adsorbed cell) was about 100 to150% higher than that of the original after washing with 0.1 mol L^{-1} H_2SO_4 solution. The MP-adsorption yields of the third reused-resting and autoclaved cells type B were 61.8±0.7% and 63.6±0.6%, respectively, while they were only 20.6±0.8% and 24.5±0.6% for the original resting and autoclaved cells type B, respectively (Table 4). Unfortunately, the MP-adsorption capacity was decreased by about 90% after the third washing with 0.1% SDS solution (Table 4).

Sequential replacement reaction for MP-adsorption capacity

MP-adsorption capacities of both resting and autoclaved cells types A and B of *I. orientalis* No.SF9-246 in the sequential replacement reaction are shown in Figure 3. Although, the adsorption capacity decreased as the replacement was replaced, the MP-adsorption capacity was recovered after been washed with 0.1 mol L^{-1} H_2SO_4 solution. Its adsorption capacity was decreased by 70 to 80% of the initial adsorption capacity after the third reuse, but its adsorption ability could be recovered to almost 100% of the initial adsorption capacity after washing with 0.1 mol L^{-1} H_2SO_4 solution.

Effect of MP on glutathione S- transferase (GST) gene expression

I. orientalis No.SF9-246 produced GST isoenzyme, Y-1

Table 3. Elution efficiency of various types of eluent.

Issactchenkia orientalis No.SF9-246		Percentage of eluted melanoidin from melanoidin adsorbed bio-sludge in various types of eluent							
Acclimatization media	**Type of cell**	**0.1% SDS**	**0.1% Tween-80**	**0.1% Triton-X100**	**0.1 mol L^{-1} NaOH**	**0.1 mol L^{-1} Phosphate buffer**	**0.1% Cholic acid**	**0.1 mol L^{-1} H_2SO_4**	**Distilled water**
MYGP[a]	Resting cell type A	98.2±1.5	64.5 ± 2.1	65.1 ± 1.0	70.4 ±2.6	54.6 ± 1.5	55.1 ± 1.1	98.0 ± 1.8	53.6 ± 1.9
	Autoclaved cell type A	98.9±2.5	60.4 ± 2.5	60.5 ± 2.7	65.5 ± 1.6	48.4 ± 1.4	45.1 ± 2.1	88.0 ± 0.9	48.4 ± 1.8
MYGP–MP[b]	Resting cell type B	86.7±2.8	50.3 ± 1.4	50.3 ± 0.9	70.5 ± 2.1	51.6 ± 3.1	28.9 ± 0.8	85.1 ± 1.6	50.6 ± 1.5
	Autoclaved cell type B	79.0±2.5	47.3 ± 2.3	48.4 ± 1.8	65.5 ± 1.1	43.4 ± 2.3	30.6 ± 1.7	83.0 ± 0.8	44.4 ± 1.6

±, Standard deviation of 3 replicates; [a], MYGP; malt extract-yeast extract-glucose-peptone broth (Difco Laboratories, 1967); [b], MPGP-MP; MYGPB (Difco Laboratories, 1967) containing MP.

Table 4. MP-adsorption efficiency of *I. orientalis* No.SF9-246 cell after re-washed with various kinds of eluent solution (%).

Issactchenkia orientalis No.SF9-246		Melanoidin adsorption yield of *Issactchenkia orientalis* No.SF9-246cell after washing with various types of eluent (%)											
Acclimatization media	**Type of cell**	**Raw yeast cell**	**Adsorption yield after washing with 0.1 mol L^{-1} NaOH**			**Adsorption yield after washing with 0.1 mol L^{-1} H_2SO_4**			**Adsorption yield after washing with 0.1 mol L^{-1} Phosphate buffer (pH 6)**			**Adsorption yield after washing with 0.1% SDS**		
			1st	**2nd**	**3rd**	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**
MYGPB[a]	Resting cell type A	27.8±1.3	26.5±0.5	5.4±0.2	11.7±0.5	42.5±0.6	51.1±0.5	56.0±0.6	26.5±0.2	6.0±0.1	11.7±0.3	26.5±0.5	3.0±0.1	7.0±0.2
	Autoclaved cell type A	25.3±0.8	25.9±0.5	6.0±0.3	7.0±0.3	42.1±0.5	53.1±0.6	58.9±0.7	25.9±0.4	2.4±0.1	3.5±0.1	25.9±0.5	3.3±0.1	3.8±0.1
MYGPB-MP[b]	Resting cell type B	24.5±0.5	29.1±0.6	3.3±0.1	14.3±0.3	44.6±0.6	54.3±0.6	63.6±0.6	29.1±0.3	1.5±0.2	10.5±0.3	29.1±0.4	8.4±0.2	9.6±0.2
	Autoclaved cell type B	20.6±0.8	20.5±0.7	4.2±0.2	13.7±0.4	37.1±0.5	56.1±0.6	61.8±0.7	20.5±0.4	3.0±0.1	14.6±0.3	20.5±0.5	3.3±0.1	5.0±0.2

±, standard deviation of 3 replicates; [a], MYGPB: Malt extract-yeast extract-glucose-peptone broth (Difco Laboratories, 1967); [b], MPGPB-MP: MYGPB (Difco Laboratories, 1967) containing MP.

and Y-2 in both MYGPB and MYGPB-MP medium. It was confirmed by the lanes of both GST Y-1 and GST Y-2 gene that was expressed on the agarose gel (Gel chromatography) as shown in Figure 4. But the size of GST Y-1 gene lane of the culture cultivated in the medium containing MP was larger than that in the medium without MP. Also, the strain did not show any difference on the size of GST Y-2 gene lane production when it was cultivated in MYGPB and MYGPB-MP (Figure 5).

Properties of *I. orientalis* No.SF9-246 cell cultivated in synthetic medium containing MP

The strain cultivated on SWW and SWW-MP generated high biomass yields of 43.6±2.5 and 30.9±2.1 g L^{-1}, respectively within 7 days of culture (data not shown). The cells that grew on SWW and SWW-MP did not show any difference on the chemical composition. The protein, lipid and ash content of the cell that grew on SWW-MP were 36.38±1.12, 0.42±0.05 and 6.67±1.23%, respectively (Table 5).

DISCUSSION

Both living (resting) and dead (autoclaved) cells of

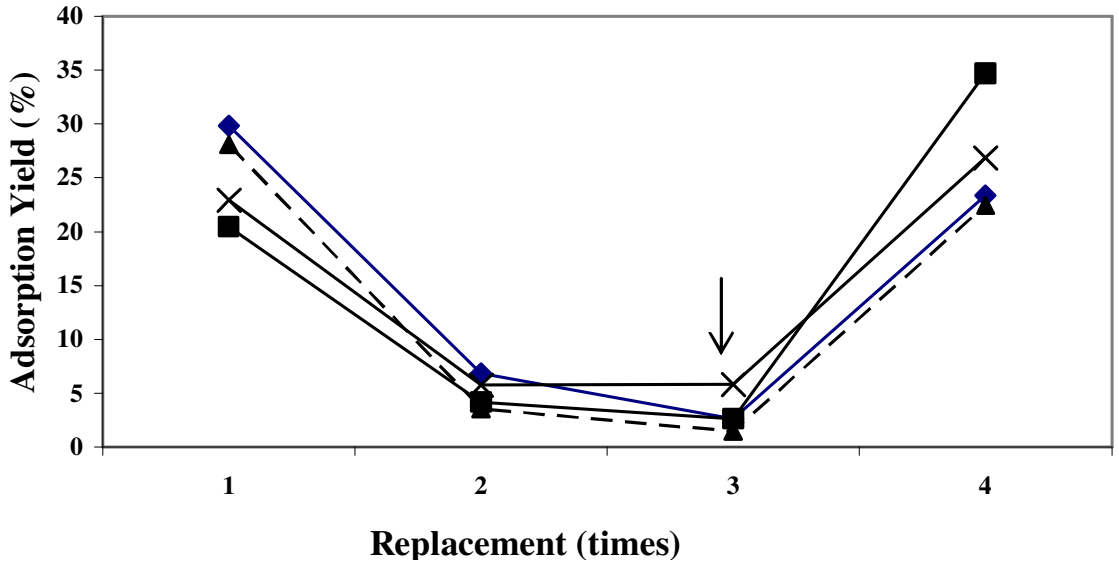

Figure 3. Adsorption yield on the successive decolorization with the reuse of *I. orientalis* No.SF9-246. (■), resting cell cultivated with MP; (◆), without MP; (▲), autoclaved cell cultivation with MP (✕) and without MP; arrow shows washing with 0.1 mol l^{-1} H_2SO_4 solution.

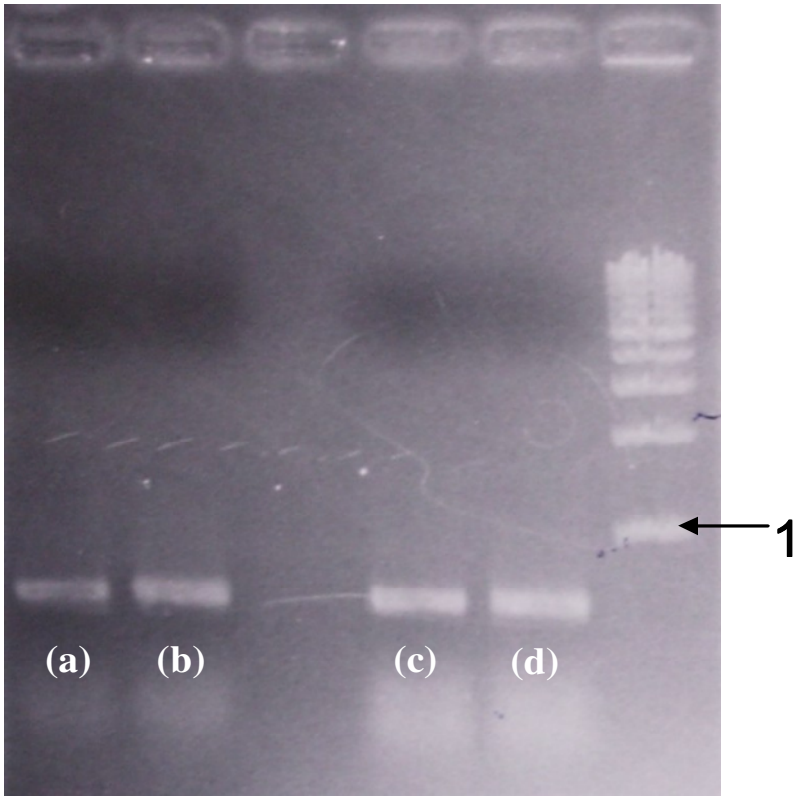

Figure 4. mRNA selective PCR of GST Y-1 and GST Y-2 (25 cycles). (a) and (b), lane of GST Y-1; (c) and (d), lane of GST Y-2.

I. orientalis No.SF9-246 (Tondee et al., 2008) could be used as the MP-adsorbent and their MP-adsorption abilities were not influenced by MP during the cultivation step. Unfortunately, the cell cultivated in the medium

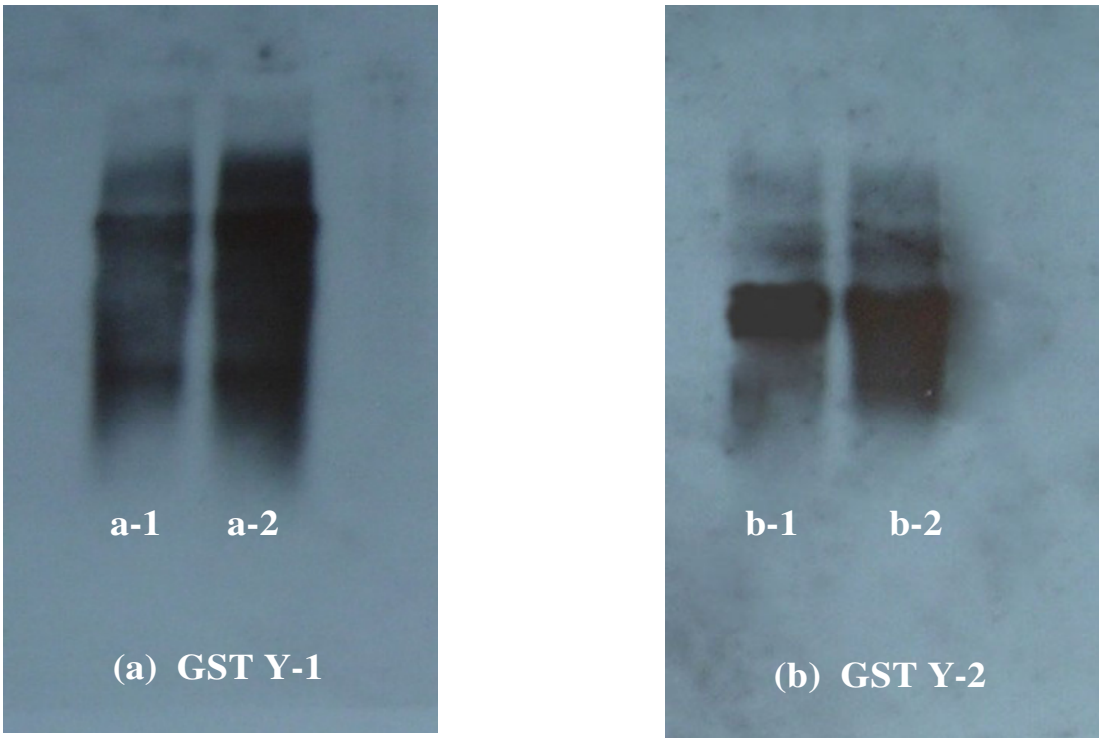

Figure 5. Northern blotting analysis. 2 µg of total RNA extracted from *I. orientalis* No.SF9-246 cells that was cultivated in medium contained with melanoidin pigment (2) and without melanoidin pigment (1) were used for analysis. A, GST Y-1; b, GST Y-2.

Table 5. Chemical composition of *I. orientalis* No.SF9-246 cell and the other fugal strains.

Type microorganism	Chemical composition				Reference
	Total nitrogen (%)	Crude protein (%)	Lipid (%)	Total ash (%)	
Issactchenkia orientalis No.SF9-246[a]	5.82±0.18	36.38±1.12	0.42±0.05	6.67±1.23	-
Saccharomyces. cerevisiae	-	35.50	0.004	0.16	Ojokoh and Uzeh (2005)
Penicillium chrydosporium	5.42	33.90	-	8.10	Uysal et al. (2002)
Hansenula sp.	8.10	50.60	1.00	3.00	Shojaosadati et al. (1999)

[a] The strain was cultivated in synthetic wastewater for 7 days.

containing MP was about 15% less than that of the cell cultivated in the medium without MP because, some MP-adsorption sites on the surface of the cell were filled up with MP during cultivation step. This phenomenon was similar to the previous work on the MP-adsorption mechanism of *Acetogenic bacteria* BP 103 (Sirian-untapiboon et al., 2004a; Sirianuntapiboon and Prasertsong, 2008). However, the adsorbed-MP was easily eluted from the deteriorated cell (MP-adsorbed cell) by washing with diluted-SDS or diluted-H_2SO_4 solutions, but it was hardly eluted by washing with distilled water. This might be the advantage of the strain to be reused as the MP-adsorbent for several times (Sirianuntapiboon and Prasertsong, 2008). Even, the diluted-SDS solution showed the highest MP-elution capacity, but the MP-adsorption capacity of the deteriorated cell was reduced by about 90% after washing with diluted-SDS solution. It might be the effect of SDS to destroy MP adsorption site on the surface of *I. orientalis* No.SF9-246 cell (Sirianuntapiboon et al., 1995; Ohmomo et al., 1988). On the other hand, the MP-adsorption capacity of the deteriorated cell was not reduced after washing with diluted-H_2SO_4 solution. Un-expectantly, the MP adsorption capacity of the deteriorated cell was increased by 100 to 150% after washing with diluted-H_2SO_4 solution. Because, the diluted-H_2SO_4 solution could remove or elute all the adsorbed-matters (positive charge) from MP-adsorption

sites resulted to increase free MP-adsorption sites (Sirianuntapiboon and Prasertsong, 2008). Also, the living cell showed a MP-adsorption yield of about 15 to 20% higher than the dead (Autoclaved) cell. It might be due to the effect of MP-degradation metabolism of living cell to increase or stimulate the MP-adsorption capacity (Sirianuntapiboon et al., 1995; Sirianuntapiboon et al., 2004a, b). This could be confirmed by the detection of intracellular MP-degradation enzyme from MYGPB-MP-cultivated cell. Moreover, the MP-decolorizing enzyme production was induced by MP. For the electron microscopic study, *I. orientalis* No.SF9-246 cell that was incubated in MYGPB-MP for 7 days became dark-brown and remained so after been washed several times with 0.1 mol L^{-1} phosphate buffer (pH 6). The electron dense materials were detected in the cytoplasm and around the cell membrane by transmission electron microscope, while the MYGPB-cultivated cell was colorless and did not show any electron dense materials. The electron dense materials in the cytoplasm and around the cell membrane might be composed of MP that had been adsorbed and absorbed (Sirianuntapiboon et al., 1995; Ohmomo et al., 1988). To confirm the stated suggestion on the MP adsorption-absorption mechanism, it was found that, the MP was accumulated onto cell wall of the 2 days culture. Unfortunately, it could not be detected in the other parts of the cell (cell membrane, endoplasmic reticulum and Golgi body). Anyways, it could not be confirm that MP did not accumulate in the other part of the cell, because, the experiment was done with only 2 days culture (log phase state). Then, it was recommended that the strain should be cultivated within longer than 2 days to observe various growth states (lag phase, stationary phase and declined phase) to observe MP-accumulation mechanism. However, the electron dense materials of the 7 days MP-cultivated cell were disappeared after re-cultivation in the media without MP for 4 days. Also, MP-decolorization activity was detected from the cell free extract of *I. orientalis* No.SF9-246 culture. It could be concluded that, the adsorbed-MP was decomposed by the intracellular MP-decolorizing enzyme (Sirianuntapiboon et al., 1995). For the mentioned results, it appeared that the mechanism for decolorizing MP involved: that the MP was absorbed into the cells in the middle of growth; log and early stationary phases (Metcalf and Eddy, 2004) and the absorbed-MP was intracellularly accumulated in the cytoplasm and near the cell wall. Later, the absorbed-MP was decomposed by intracellular MP-decolorizing enzyme. This phenomenal was similar to our previous works on the MP decolorization mechanism of *Rhizoctonia* sp. D-90 (Sirianuntapiboon et al., 1995; Sirianuntapiboon et al., 1998).

The strain also showed the other advantage that glutathione S-transferase (GST) enzyme was generated as the by-product during MP treatment. Both GST Y-1 and GST Y-2 genes were expressed during the cultivation in both MYGPB and MYGPB-MP. Moreover, the expression of GST Y-1 gene was induced by MP. Many researchers reported that, MP was a kind of inhibitor to repress the growth of microorganisms such as mold, bacteria and yeast (Sirianuntapiboon et al., 1995; Sirianuntapiboon and Prasertsong, 2008). It could therefore be suggested that, GST Y-1 might play a role to detoxify MP. Also, it was reported that GST played a major role in the drug resistance and carcinogen-detoxification (Tamaki et al., 1989; Tamaki et al., 1999; Collinson and Grant, 2003). But, the relevant data for the determination of the detoxify MP by GST Y-1 was not collected in this study. It is therefore recommended that, further research regarding the toxicity of MP on the growth of *I. orientalis* No.SF9-246 and the induction mechanism of MP on the GST Y-1 production be conducted to further understand the relationship between GST Y-1 and MP. Moreover, the biomass production of the strain with medium containing MP was 40% higher than that with medium without MP, because MP contained large amount of nitrogen and phosphorus which resulted to the stimulation of the growth of *I. orientalis* No.SF9-246 (Ricci et al., 1987; Cristiani-Urbina et al., 2000; Moeini et al., 2004). The percentage of crude protein of *I. orientalis* No.SF9-246 cell was 36.38±1.12%. It was almost similar to the other yeast strain, *Saccharomyces cerevisiae* that is commonly used as a protein source (single cell protein: SCP). Then, it can be suggested that the biomass of *I. orientalis* No.SF9-246 could be used as the protein source for feed and food (Ojokoh et al., 2002; Uysal et al., 2002).

Conclusions

I. orientalis No. SF9-246 cell showed both MP-adsorption and MP-degradation abilities. The MP degradation ability was induced by MP, while MP did not influence the MP adsorption ability. Then, the MYGPB-cultivated living cell showed the highest MP-adsorption yield of 27.8±1.3%. The deteriorated cell (MP-adsorbed cell) could be reused after washing with diluted-H_2SO_4 solution. Un-expectedly, MP-adsorption capacity of the deteriorated cell was increased by 100 to 150% after washing with diluted H_2SO_4. The harvested-biomass from MP treatment system could be used as the protein source for animal feed due to the high protein content of 36.38±1.12%. The other advantage of this strain was GST isoenzyme and Y-1 and Y-2 (GST Y-1 and GST Y-2) products generated during MP treatment.

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