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Biodegradation of oil wastewater by free and immobilized *Yarrowia lipolytica* W29

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Abstract

The ability of *Yarrowia lipolytica* W29 immobilized by calcium alginate to degrade oil and chemical oxygen demand (COD) was examined. The degradation rules of oil and COD by immobilized cells with the cell density of 6.65×10^6 CFU/mL degraded 2000 mg/L oil and 2000 mg/L COD within 50 h at 30°C (pH 7.0, 150 r/min), similarly to those of free cells, and the degradation efficiencies of oil and COD by immobilized cells were above 80%, respectively. The factors affecting oil and COD degradation by immobilized cells were investigated, the results showed that immobilized cells had high thermostability compared to that of free cells, and substrate concentration significantly affected degrading ability of immobilized cells. Storage stability and reusability tests revealed that the oil degradation ability of immobilized cells was stable after storing at 4°C for 30 d and reuse for 12 times, respectively, the COD degradation rate of immobilized cells was also maintained 82% at the sixth cycle. These results suggested that immobilized *Y. lipolytica* might be applicable to a wastewater treatment system for the removal of oil and COD.

Key words: *Yarrowia lipolytica*; biodegradation; oil wastewater; calcium alginate
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Introduction

Oil and its derivative from oil manufacturing plants and restaurants have received attention because of their widespread use. Disposal of this wastewater into environment causes serious problems due to its high oil content, chemical oxygen demand (COD) and color. Microbial degradation of oil wastewater is a concern in recent years. A variety of microorganisms such as bacteria, molds, and yeasts, have been shown to be capable of completely degrading oil wastewater (Ammar *et al*., 2005; Dhouib *et al*., 2006; Erguder *et al*., 2000; Ettayebi *et al*., 2003; Kissi *et al*., 2001). Zheng *et al*. (2005) utilized oil as a substrate for biomass production of yeast isolate, and Papanikolaou *et al*. (2008) reported using oil mill wastewater (OMW)-based media producing citric acid, the phenolic compounds of OWM were decreased simultaneously and a remarkable decolorization was observed.

Immobilization of whole cells for the degradation of different compounds in wastewater has several advantages: (1) to provide high activity, yield, and good operational stability; (2) to separate cell mass from bulk liquid for possible reuse (Reischwitz *et al*., 1995). Immobilization technology has been widely applied for degradation of many compounds, such as quinoline (Wang *et al*., 2002), Phthalate (Patil *et al*., 2006), sulfate (Kuo and Shu, 2004), dimethyl sulfoxide (Murakami-Nitta *et al*., 2003). Calcium-alginate (Ca-alginate) has been widely employed for immobilization of enzymes or whole cells since it is less toxic than synthetic polymers, easily gelled under mild conditions and inexpensive (Gonzalez *et al*., 2001; Shimomura *et al*., 1997; Wang *et al*., 2007).

Biological treatment of salad oil and grease from food wastewater by *Yarrowia lipolytica* W29 has been studied (Wu *et al*., 2006). When concentration of oil $\leqslant$ 2000 mg/L, the removal rates of salad oil and grease from food wastewater by free cells attained 93.30% and 85.08% under the optimum conditions for 50 h, respectively. In this study, we evaluated the ability of *Y. lipolytica* W29 immobilized in Ca-alginate to degrade oil and COD in wastewater. In addition, regularity of degradation, reusability and storage stability of immobilized cells were investigated.

1 Materials and methods

1.1 Micro-organism and preparation inoculum

Yarrowia lipolytica W29 used in this study was originally isolated from Dr. Gaillardin (France). YPD medium (1% yeast extract, 2% bacterial peptone, 2% glucose, pH 7.0) was used for the preparation of inoculum. The YPD medium was inoculated with *Y. lipolytica* and incubated

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for 18 h in a shaking flask (150 r/min) at 30°C.

1.2 Immobilization of whole cells

Pre-inoculum wet cells 4 mL were mixed with 4% sterile sodium alginate solution. The concentration of suspended cells was 6.65×10^6 CFU/mL based on Hutchison blood cell counting (Shen *et al*., 2000). The mixture was dropped into sterile 0.1 mol/L $CaCl_2$ solution. The droplets gelled in contacted with Ca^{2+} cations forming calcium alginate beads which were left to harden for 1 h at room temperature, washed twice with distilled water after cross-linking for 24 h at 4°C (Qu and Li, 2000).

1.3 Growth of *Y. lipolytica* in oil wastewater

The salad oil wastewater was artificial made by adding salad oil (Jinlongyu, Shenzhen, China) in water. Activated *Y. lipolytica* cells were inoculated into 150 mL of 2000 mg/L sterile salad oil wastewater and incubated in a shaking flask (150 r/min) at 30°C. Growth was monitored by measuring absorbance at 600 nm at regular intervals of 5 h using 756-type spectrophotometer (Shanghai Spectrum, China), based on the method of Zhao and He (2002).

1.4 Oil and COD degradation by free and immobilized *Y. lipolytica*

Either immobilized or free *Y. lipolytica* (6.65×10^6 CFU/mL) was incubated for 50 h at 30°C (pH 7.0) with 2000 mg/L oil and 2000 mg/L COD concentration in 500 mL flash (150 r/min) to adjust total volume to 150 mL. Oil and COD degradation rates were measured every 10 h and up to 50 h.

1.5 Effect parameters

The effect of temperature on oil degradation by both free and immobilized cells of *Y. lipolytica* was studied. Free and immobilized cells degraded initial concentration of 5000 mg/L oil wastewater with pH 7.0, 150 r/min for 50 h at a temperature range from 15 to 40°C. The effect of original concentration of oil and COD on the degradation of oil and COD by immobilized cells was studied. Immobilized cells embedded with Ca-alginate were grown in oil and COD wastewater (pH 7.0) for 50 h at 30°C, 150 r/min with different initial oil and COD wastewater.

1.6 Analytical methods

Oil concentration was determined according to the gravimetric method (National Standard CJ/T57-1999, China). Oil-degradation rate (%) was defined as the amount of oil degraded versus the amount of initial oil. COD was determined according to the dichromate titration (National Standard CJ/T56-1999, China). The COD degradation efficiency was defined as the amount of COD decreased versus the amount of initial COD. All the experiments were performed in triplicate and data presented as means ± SE.

1.7 Microscopic examination

Immobilized cells were fixed with 1% glu-taraldehyde in cocadylic buffer, dehydrated and prepared for observation with S570 scanning electron microscope (Hitachi Ltd., Japan) according to a standard procedure (Van Neerven *et al*., 1990).

1.8 Statistical analysis

In this study, one-way ANOVA was separately used in comparing differences between oil degradation rates to the cycle number and COD degradation rates to the cycle number; two-way ANOVA was used in all other statistical analysis. Statistical analysis was performed using software SPSS 11.0 (SPSS Inc., USA). All tests were considered significant at $\alpha = 0.05$ level.

2 Results and discussion

2.1 Growth of *Y. lipolytica*

The growth curve of *Y. lipolytica* under the experimental conditions is illustrated in Fig. 1. It shows that there were mainly four phases: (1) lag phase, which lasted from 0 to 5 h and the absorbance was 0.0225; (2) logarithmic phase, which lasted from 5 to 20 h, and the absorbance increased from 0.0225 to 1.119; (3) stationary phase, which lasted from 20 to 35 h, and the absorbance was about 1.1; (4) death phase, the absorbance decreased after 35 h. This experimental result showed that *Y. lipolytica* was capable of using salad oil as the sole source of carbon, nitrogen and energy, and *Y. lipolytica* presented remarkable growth in salad oil wastewater.

Previous works indicated that *Y. lipolytica* could be used to treat different wastewater, such as: OMW, palm oil mill effluent (POME), and caprolactam waste liquor (De Felice *et al*., 1997; Oswal *et al*., 2002; Johnson *et al*., 1994). These studies showed that some *Y. lipolytica* strains were good candidates to reduce COD level, increase biomass production, and induce some enzyme and metabolites production such as lipase and citric acid (Lanciotti *et al*., 2005). Scioli and Vollaro (1997) have used a strain of *Yarrowia lipolytica* ATCC 20255 to treat OMW containing high COD load of 100–200 g/L. *Y. lipolytica* ATCC 20255 was capable of reducing the COD by 80% in 24 h, produced useful biomass of 22.45 g/L and enzymes. Some stains of *Y. lipolytica* also were adapted to grow in industrial fat and they could uptake fatty acids (Papanikolaou *et al*., 2002; Papanikolaou and Aggelis, 2003).

2.2 Oil and COD degradation by the free and immobilized *Y. lipolytica*

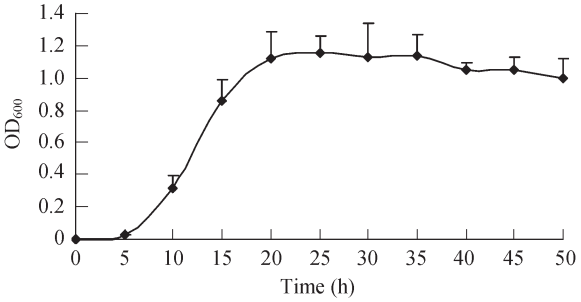

Fig. 1 Growth curve of *Yarrowia lipolytica* W29 in salad oil wastewater.

Biodegradation of oil, COD by immobilized and free cells is shown in Fig. 2. The oil and COD degradation rates were increased with time extension for both free and immobilized cells. Statistical analyses suggested that the rate of oil degradation differed among time points (F = 33.70, P < 0.0001), both free and immobile cells showed similar oil degradation rates (F = 1.2193, P > 0.05), and there was significant interaction between time and treatment (F = 2.9831, P = 0.0312). The rate of COD degradation differed among time points (F = 15.499, P < 0.0001), the COD degradation rates by free and immobilized cells (or treatment) were significantly different (F = 24.0272, P < 0.0001) with immobilized cells showing higher degradation rates and there was no significant relationship between time and treatment.

Although the degradation rates by free and immobilized cells varied with substrate types, within 20 h, the oil and COD degradation rates by immobilized cells were higher than those of free cells. This can be owing to that during this initial period, the calcium alginate acted as oil and COD adsorbent as well as immobilized carrier.

Comparison of oil and COD removal efficiencies by either free or immobilized cells indicated that the efficiency of removal of oil and COD by free cells was 93.3% and 66.95% respectively, and the degradation rates of oil and COD were significantly different (F = 32.6424, P < 0.0001). Similarly, the efficiency of removal of oil and COD by immobilized cells was 82.2% and 88.2%, respectively, and the rates also were significantly different (F = 33.3538, P < 0.0001). Results indicated that substrate type was an important factor to affect degradation ability of *Y. lipolytica*.

Our findings of the degradation of wastewater containing high concentrations of oil and COD by free *Y. lipolytica* were similar with those results in previous publications (Lanciotti *et al*., 2005; Oswal *et al*., 2002; Papanikolaou *et al*., 2002; Scioli and Vollaro, 1997). However, they only focused on the application of free *Y. lipolytica* in the wastewater system. In this study, it is significant report to note the capability of *Y. lipolytica* immobilization for wastewater treatment.

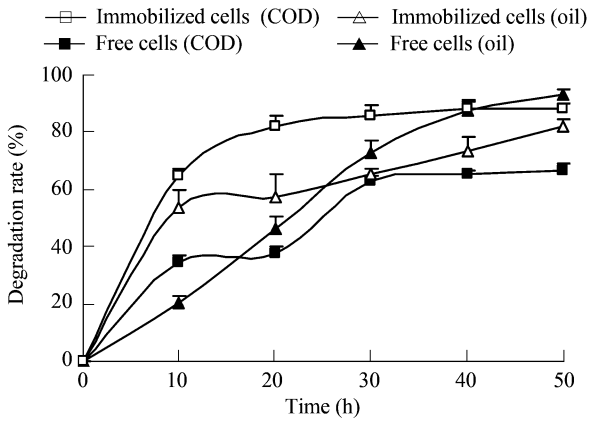

Fig. 2 Oil and COD degradation by the free and immobilized cells under optimum conditions.

2.3 Effect of temperature on oil degradation

The results in Fig. 3 showed that temperature significantly affected oil degradation rate by *Y. lipoltyica* (F = 33.1963, P < 0.0001), with the highest oil degradation level by free and immobilized cells at 30°C. When the temperature changed from 15 to 40°C, free and immobilized cells did not differ in their ability to degrade oil (F = 1.5623, P > 0.05) and there was significant interaction between temperature and treatment (free versus immobile) (F = 6.6727, P < 0.001). These results indicated that the optimum temperature of oil degradation by free and immobilized cells was the same. However, the immobilized cells had a wide temperature range than that of free cells. Suitable temperatures for oil degradation by free and immobilized cells range from 25 to 30°C and from 25 to 35°C, respectively. In addition, when the temperature was increased from 30 to 40°C, the oil degradation rates of free and immobile cells were reduced by 38% and 7%, respectively (Fig. 3). The changes in the degradation rates of immobile cells were less sensitive than that of free cells, suggesting the higher thermal stability of the immobilized cells.

2.4 Effect of oil and COD concentration on oil and COD degradation by immobilized cells

Factors affecting oil degradation of immobilized cells such as pH, temperature and agitation have been studied (Wu and Wan, 2008). To further determine the effect of oil and COD concentration on oil and COD degradation, immobilized cells were grown in oil and COD wastewater with different initial oil and COD concentration under the optimum conditions. The results are shown in Fig. 4.

The degradation efficiency of oil by immobilized cells decreased with the increasing of the initial oil concentration, this was similar with the effect of oil concentration on oil degradation by free cells (data not shown). When the concentration of oil ⩽ 3000 mg/L, the oil degradation rate by immobilized *Y. lipolytica* maintained above 80% and displayed better oil removal efficiencies. However, when concentration of oil > 3000 mg/L, the oil degradation rate of immobilized cells was lower than 60%. This suggested that the immobilized *Y. lipolytica* was more effective the treatment of oil wastewater, which had less substrate

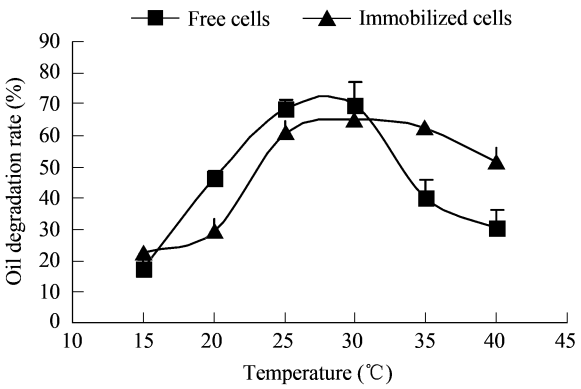

Fig. 3 Effect of temperature on oil degradation by free and immobilized cells.

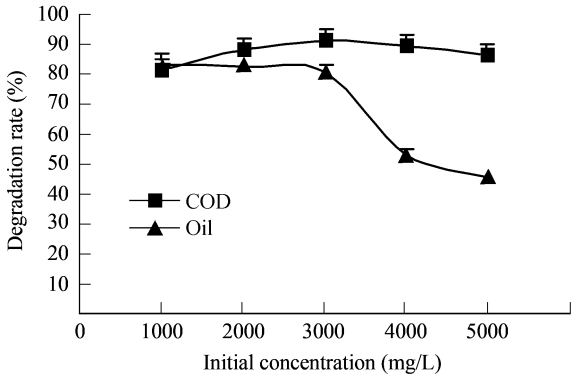

Fig. 4 Effect of initial oil and COD concentration on oil and COD degradation by immobilized cells.

concentration. On the contrary, the degradation efficiencies of COD by immobilized cells were less affected by higher COD concentration, suggesting that immobilized *Y. lipolytica* had high tolerance to changes in COD concentrations. These results verified that *Y. lipolytica* was capable of reducing the high concentration COD wastewater. Since the degradation efficiencies of oil and COD were above 80% at 30°C, 150 r/min with 2000 mg/L oil and COD concentration in 50 h reaction, the following experiments were performed under the same conditions.

2.5 Repeated oil and COD degradation by reuse of the immobilized cells

The reuse of immobilized cells might be advantageous because it can decrease waste of cells, save time, and cut down cultivation cost. The repeated oil and COD wastewater degradation by reuse of the immobilized cells were performed under the optimum conditions. The immobilized cells were added into 150 mL of 2000 mg/L oil and COD wastewater respectively. After 50 h reaction, the previous 2000 mg/L oil and COD wastewater were decanted; beads were washed with sterile water and transferred into 150 mL of the fresh 2000 mg/L oil and COD wastewater. At the same time, the weight of Ca-alginate beads with immobilized cells for the degradation of oil was measured during each cycle.

The results in Fig. 5 indicated that cycle number significantly affected both oil ($F = 5.3333$, $P < 0.001$) and COD ($F = 18.1877$, $P < 0.0001$) degradation by immobilized cells. For the oil wastewater, the immobilized cells could be reused for a maximum of 12 cycles and total 2357.1 mg oil were degraded, 77% degradation rate was found even at the 12th treatment. For the COD wastewater, the immobilized cells could be reused for a maximum of 6 cycles and total 1475.1 mg COD were degraded. A decrease in the degradation capacity was noted thereafter (Fig. 5a). Cycle number also was a significant factor to affect weight of Ca-alginate beads ($F = 13.9058$, $P < 0.0001$) in oil wastewater treatment (Fig. 5b), the weight of immobilized beads rose consistently with the increased cycle number before 10 cycles. Thereafter, the weight of beads decreased, especially when the immobilized cells were reused the 13th cycle, the weight of beads and oil degradation capacity were sharply decreased and finally

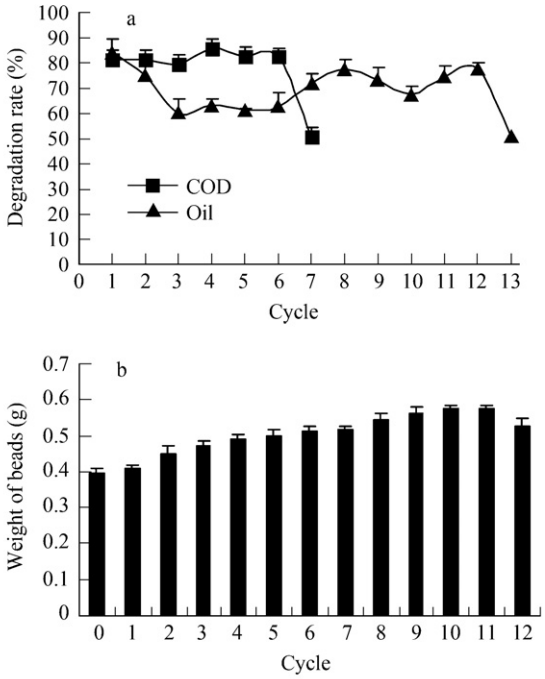

Fig. 5 Repeated oil and COD degradation by immobilized cells (a) and the weigh vary of Ca-alginate beads (b) in the repeated treatments of oil wastewater.

led to cell leakage from these beads (data not shown). These results indicated that the immobilized cells might grow on oil and COD as the carbon source during the repeated oil, COD degradation.

2.6 Storage stability of oil degradation activity

The stability during long-term storage and operation is an essential factor for practical application of immobilized cell system. In order to investigate the storage stability of oil degradation activity, the free and immobilized cells were stored in 150 mL distilled water at 4°C. After storage of the mixture for 0, 7, 14, 21, and 30 d, salad oil was added to the mixture to give 2000 mg/L. Thereafter, oil degradation was performed under the optimum conditions.

As shown in Fig. 6, immobilized and free cells did not differ in their storage stability during the time tested ($F = 0.0011$, $P > 0.05$), the degradation rates of oil both by immobilized and free cells slightly decreased with the extension of storage time ($F = 8.5786$, $P < 0.001$).

The degradation rates of oil by immobilized and free

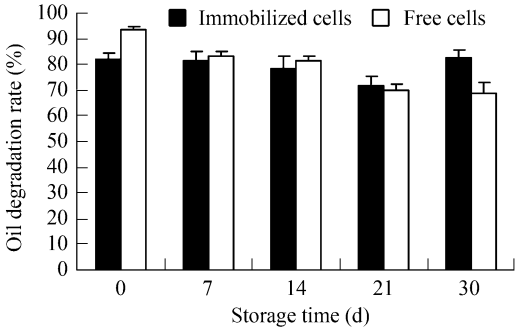

Fig. 6 Storage stability of oil-degradation activity.

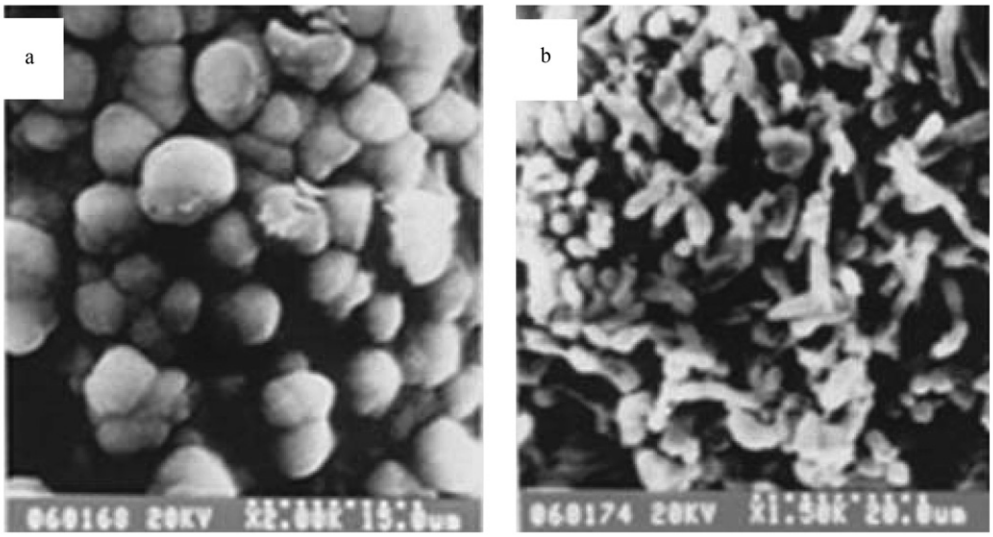

Fig. 7 Scanning electron microscope of blank Ca-alginate bead (a) and cells (b) immobilized in Ca-alginate after degrading oil wastewater 50 h under optimum conditions.

cells maintained at 82% and 68% after being stored for 30 d at 4°C, respectively, suggesting that storage stability of immobilized cells is better than free cells. In addition, the degradation efficiency was above 60% after reuse for 12 cycles by immobilized cells being stored 30 d at 4°C (data not shown), the immobilized cells maintained physiological stability even at the 12th reaction, and the Ca-alginate beads had high mechanical strength and operational stability.

2.7 Scanning electron microscopy observing immobilized cell

Figure 7 shows the scanning electronic micrographs of blank Ca-alginate beads and cells immobilized in the Ca-alginate after treating oil wastewater 50 h under optimum conditions. The blank Ca-alginate beads were scabrous on interior surface, and carrier had multiple porous structures that profited the diffusion of oxygen, substrates and metabolites (Fig. 7a). Immobilized cell growth could be locally intense after degrading oil wastewater for 50 h, the cells density was high, and cells formed clump and were present in the void space of the porous matrix (Fig. 7b). The result further suggested that Ca-alginate might be come to ideal carrier of immobilized *Y. lipolytica*. Moreover, the carrier had enough space to support cell growth.

3 Conclusions

The following results suggested that immobilized *Y. lipolytica* by Ca-alginate might be applicable to a wastewater treatment system for the removal of oil and COD. (1) the COD degradation was comparatively higher from immobilized cells in Ca-alginate beads than that by free cells; (2) the oil degradation by immobilized and free cells was similar; (3) the thermostability, reusability, and storage ability of immobilized cells for wastewater treatment were retained at a higher level.

Acknowledgments

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