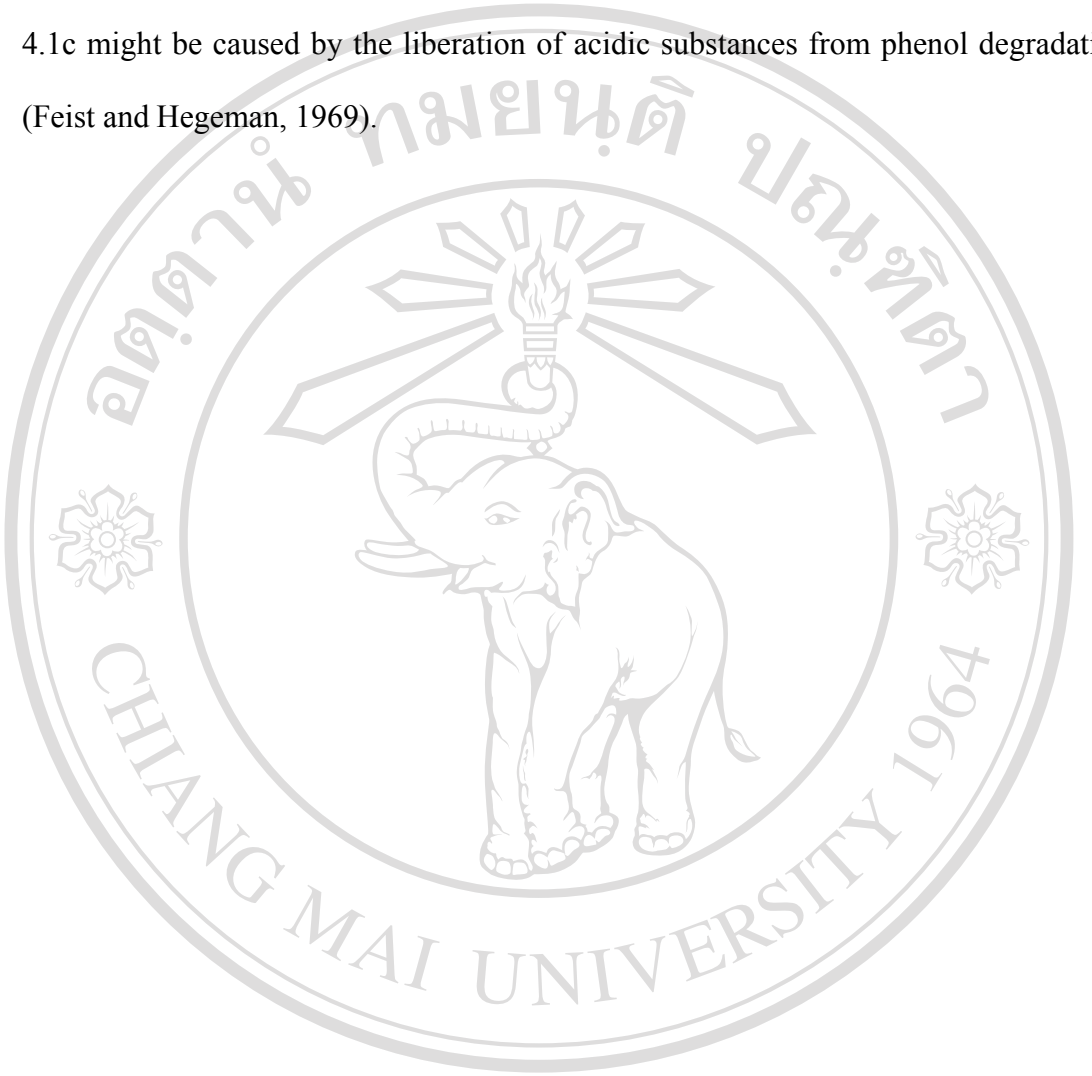
CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 The selection of optimal temperature for phenol degradation

The investigation on the effect of temperatures on phenol degradation by *C. tropicalis* CMU 10 was conducted in the MS medium containing 100 mg/l phenol at the temperature range from 20 to 45°C. Phenol degradation efficiency of phenol could be possible at a temperature range between 30-42°C. The optimal temperature was at 37°C (Figure 4.1a). Therefore, 37ºC was chosen for the next experiments on phenol degradation. This yeast could therefore be classified as a thermotolerant type of microorganism. Completed degradation at 25-40°C was achieved when aerobic granule of the yeast was cultivated (Adav *et al*., 2007). *Klebsiella oxytoca* could degrade phenol at 25-42 °C (Shawabken *et al.,* 2007). Therefore, *C. tropicalis* CMU 10 showed a wider range of temperature for phenol degradation than those previously reported for yeast. The increase or decrease in temperature may also influence the rate of phenol degradation. The biomass concentration obtained at 37°C was the highest (Figure 4.1b) while no detectable cell growth was observed at 45°C. A shift to higher temperature clearly affected the biomass concentration. The decline of microbial

activity beyond the optimum temperature could be the result of enzyme denaturation (Shuler and Kargi, 1992; Suthersen, 1999). The decrease in pH as observed in Figure 4.1c might be caused by the liberation of acidic substances from phenol degradation (Feist and Hegeman, 1969).



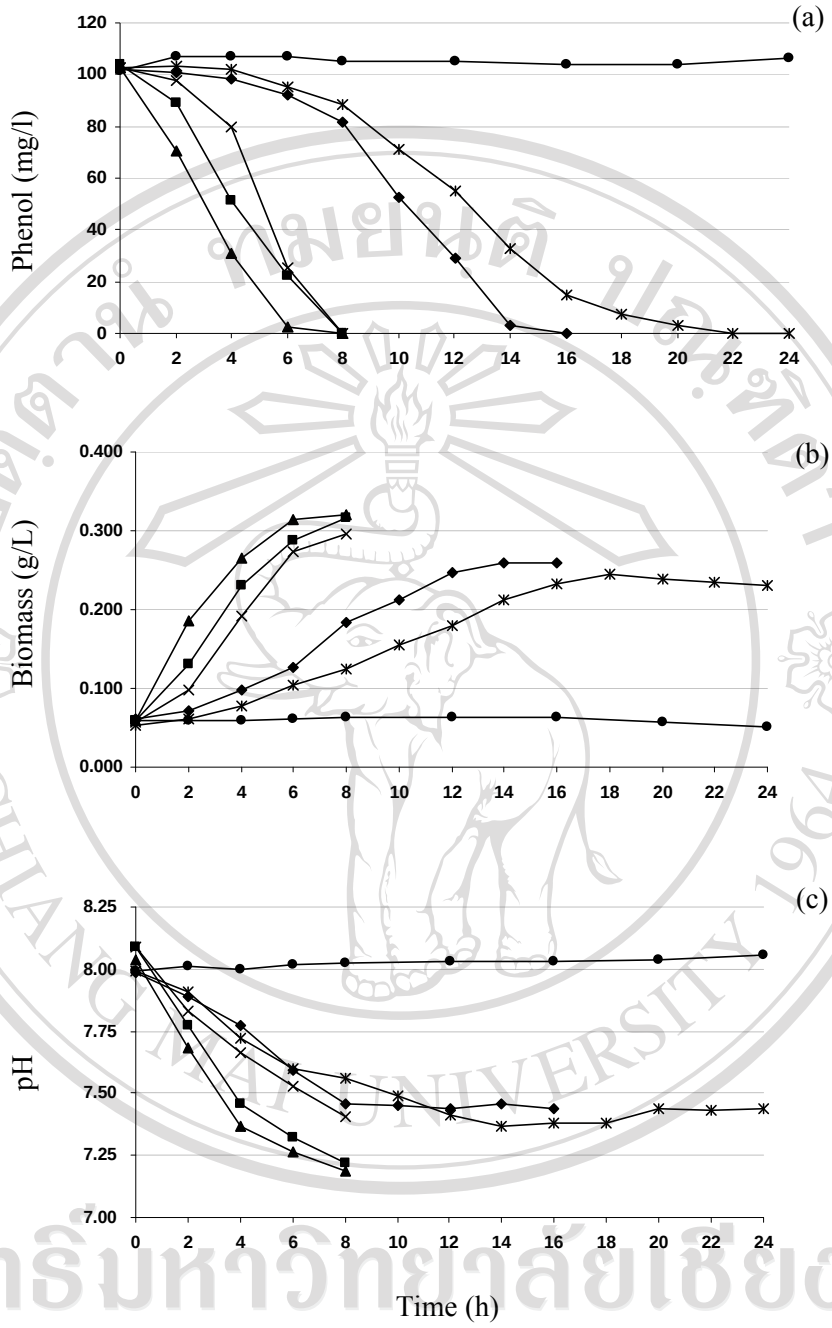


Figure 4.1 The effect of temperature on phenol degradation by *C. tropicalis* CMU 10 on the;

(a) phenol concentration,

(b) biomass concentration,

(c) pH level.

4.2 The effect of initial phenol concentration

As shown in the previous result, the optimal temperature for phenol degradation was 37ºC and therefore this temperature was employed in the investigation on the effect of phenol concentration on phenol degradation in the MS medium containing 350 to 1000 mg/l phenol (Figure 4.2a). The yeast strain was able to degrade phenol completely up to 1,000 mg/l in the MS medium alone. Phenol inhibitory level for the degradation by *C. tropicalis* CMU 10 is higher than other strains as tabulated in Table 2.6. Many microbial strains capable of degrading phenol at high concentrations (> 500 mg/l) required certain supplements, such as yeast extract and peptone, to attenuate phenol toxicity (Lob and Tar, 2000; Kotresha and Vidyasagar, 2008). From the current result, the time required to completely degrade phenol was directly proportional to the initial phenol concentration in the medium. The absence of lag phase was evident in Figure 4.2b where the yeast immediately degraded the phenol between the concentration range of 350-750 mg/l. The apparent decrease in pH level in Figure 4.2c might be the result of acidic substances liberation from phenol degradation (Feist and Hegeman, 1969).

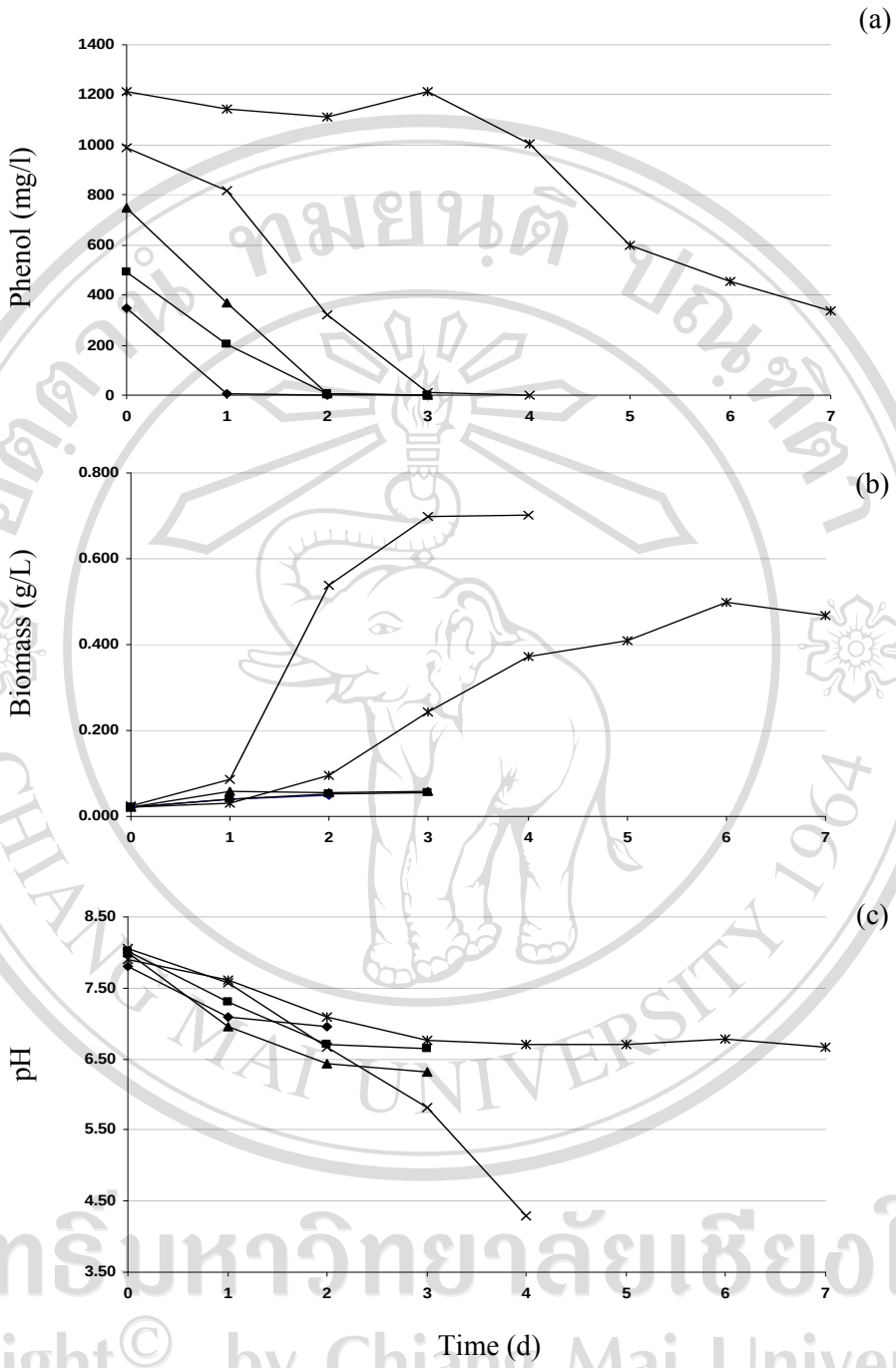


Figure 4.2 The effect of initial phenol concentration (mg/l) on phenol degradation by *C. tropicalis* CMU 10 on the**;**

(a) phenol concentration,

(b) biomass concentration,

(c) pH level.

4.3 The effect of initial pH

The investigation on the effect of pH on phenol degradation was conducted in the MS medium at different initial pH ranging from 5 to 9. The most favorable pH for the strain to achieve the maximum rate of phenol degradation was 8.0 (Figure 4.3a). More time was spent in the experiment at the initial pH of 9 and 7, whereas poor degradation was detected at pH 5 and 6. Therefore, the initial pH should be adjusted to neutral or weak alkaline condition, even though generally yeasts grow under weak acidic condition. Different initial pH values were used in the study on phenol degradation by *C. tropicalis*, ranging, for example, from 5.2-6.7 (Komárková *et al*., 2003; Juaxrez-Ramixrez, 2001; Jiang *et al*., 2005; Varga and Neujahr, 1970; Bastos *et al*., 2000). An ability of *C. tropicalis* to tolerate a wide pH range of 3-9 (Bastos *et al*., 2000) and the optimal pH of 5-6 and 7 have been reported (Chang *et al*., 1998; Adav *et al*., 2007; Kotresha and Vidyasagar, 2008). Cell biomass increased with times. The biomass produced at pH 8 was the highest (Figure 4.3b). The decrease in pH (Figure 4.3c) was due to the liberation of acidic substances from phenol degradation (Feist and Hegeman, 1969).

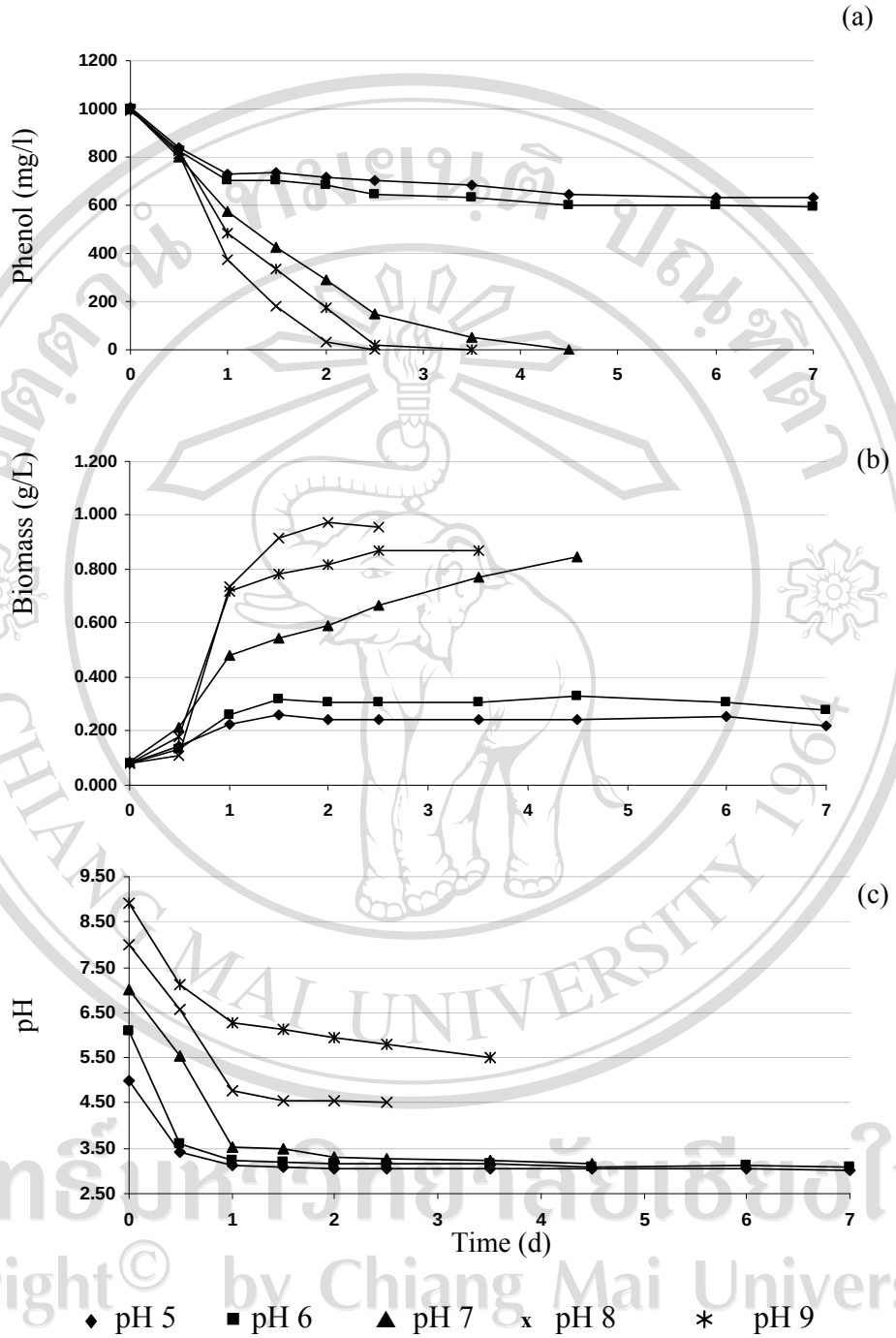


Figure 4.3 The effect of pH on phenol degradation by *C. tropicalis* CMU 10 on the;

(a) phenol concentration,

(b) biomass concentration,

(c) pH level.

4.4 The effect of glucose concentration

The investigation on the effect of glucose concentration on phenol degradation by *C. tropicalis* CMU 10 was conducted in the MS medium containing different concentrations of glucose. This monosaccharide was selected because it is a common substrate usually found in wastes of urban or agricultural origin. At the glucose concentration range of 0-0.31 mM, the yeast strain could degrade phenol without lag phase and 1,000 mg/l of phenol was entirely degraded within 4 days (Figure 4.4a). The glucose concentration during this range enhanced phenol degradation faster than the other two conditions with higher glucose concentrations (2.5 mM and 5 mM). This occurred despite an increase in cell biomass (Figure 4.4b). Ten mM glucose inhibited phenol degradation**.** Different results have been reported on the effect of glucose concentration on phenol degradation which depended on the microbial strains and the level of glucose concentrations employed. Phenol removal efficiency by the biological sludge was increased in the presence of low glucose concentration (0.28 mM) but leveled off when the concentration was between 0.7-2.8 mM (Movahedian, 2005). Two-stage growth of *C. albican* TL3 was observed in the medium containing phenol and 0.67% glucose where glucose was consumed rapidly in the first stage and subsequently phenol in the second stage (Tsai *et al.*, 2005). In our study, another possible reason for the depression of phenol degradation in the presence of higher glucose concentration might be the swift decrease in pH to the level as low as 3.07 at which phenol degradation was completely inhibited. Therefore, phenol biodegradation rate was inversely proportional to the concentration of glucose. The decrease in pH level (Figure 4.4c) might stem from the liberation of acidic substances after phenol

degradation (Feist and Hegeman, 1969). Previous studies had elucidated similar negative effects of glucose on phenol degradation by different microbial species (Bastos *et al*.; 2000a; Swindoll *et al*., 1988; Rozich and Colvin, 1986; Kar *et al*., 1996; Movahedian, 2005; Tsai *et al*., 2005). Detrimental effect of glucose on phenol degradation was investigated by Kar *et al*., (1996) who found that glucose inhibited one of the enzymes responsible for phenol degradation metabolism and prevented intracellular phenol transportation. In comparison to phenol, glucose was relatively easy to degrade and was thus more preferable by the cells. Repression of aromatic compound biodegradation by glucose had also been reported as a result of catabolite repression (Muller *et al*., 1996).

Sequential utilization of substrate is an important problem in waste treatment system whereby the preferred substrate by the microbes is consumed first and leave the pollutant behind (Goldstein *et al*., 1985). Since *C. tropicalis* CMU 10 has simultaneous substrate utilization pattern, it may serve as a potential candidate for pollution control in industrial wastewater treatment and bioremediation as it is able to degrade more than one organic pollutants at the same time.

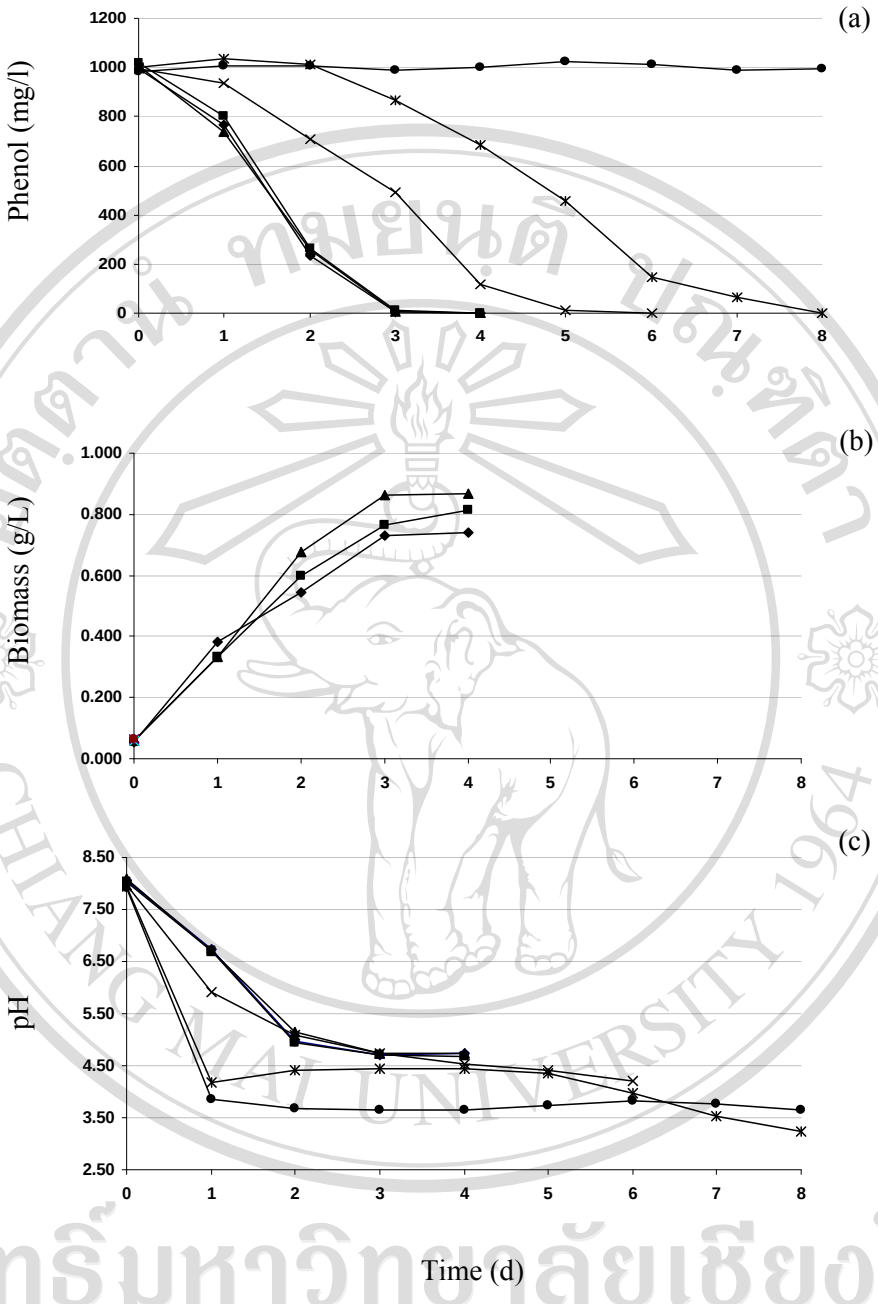


Figure 4.4 The effect of glucose concentration on phenol degradation by *C. tropicalis* CMU 10 on the;

(a) phenol concentration,

(b) biomass concentration,

(c) pH level.

4.5 The effect of organic acids concentration

The investigation on the effect of organic acids on phenol degradation by *C. tropicalis* CMU 10 was conducted in the MS medium containing different species of organic acids including citric acid, lactic acid, malic acid and succinic acid at a concentration level of 10 mM. The impact of citric and succinic acids on phenol degradation was negligible and the degradation was completed after 4 days which was equivalent to control (Figure 4.5). The complete phenol degradation with the presence of lactic acid was also possible at a slow rate in which overall consumption period was extended to 7 days. The addition of malic acid resulted in the strong inhibitory effect of phenol degradation where merely 5.26% of the phenol was degraded. Phenol degradation is commonly subjected to carbon catabolite repression induced by organic acids (Muller *et al*., 1996; Lob and Tar, 2000).

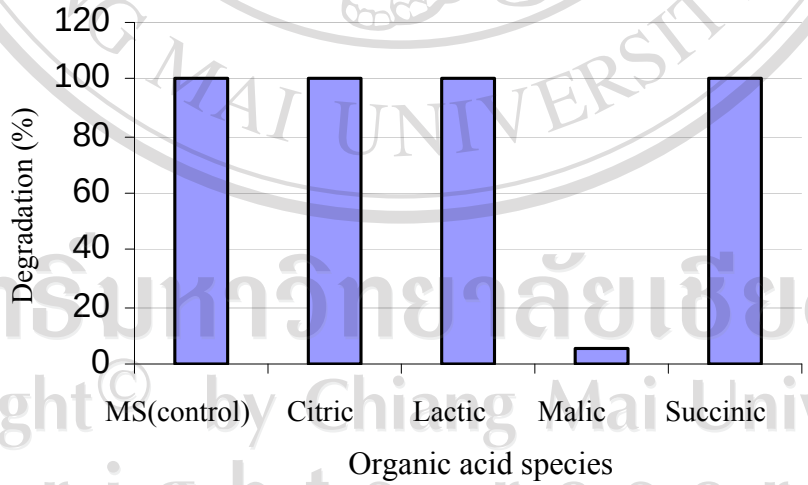


Figure 4.5 The effect of organic acid species on phenol degradation by *C. tropicalis* CMU 10.

4.6 The effect of metal ions concentration

The investigation on the effect of metal ions on phenol degradation by *C. tropicalis* CMU 10 was conducted in MS medium containing different metal ions including cobalt, copper, manganese, ferrous, ferric, nickel and zinc at a concentration level of 0.4 mM. Manganese and ferrous ions did not interfere with phenol degradation as evident from the comparison with control (Figure 4.6). Phenol was consumed completely after 4 days. Ferric and zinc ions repressed phenol degradation slightly, the degradation was completed after 5 and 6 days, respectively. Cobalt, nickle and copper ions strongly affected phenol degradation. The inhibitory effect of copper ions reported to be was much stronger than that of zinc on phenol consumption by *Acinetobacter calcoaceticus* AH strain (Nakamura and Sawada, 2000). However, Metal ions such as Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} at low concentrations might stimulate and enhance the rate of phenol degradation by *Pseudomonas aeruginosa* MTCC 4996 (Kotresha and Vidyasagar, 2008).

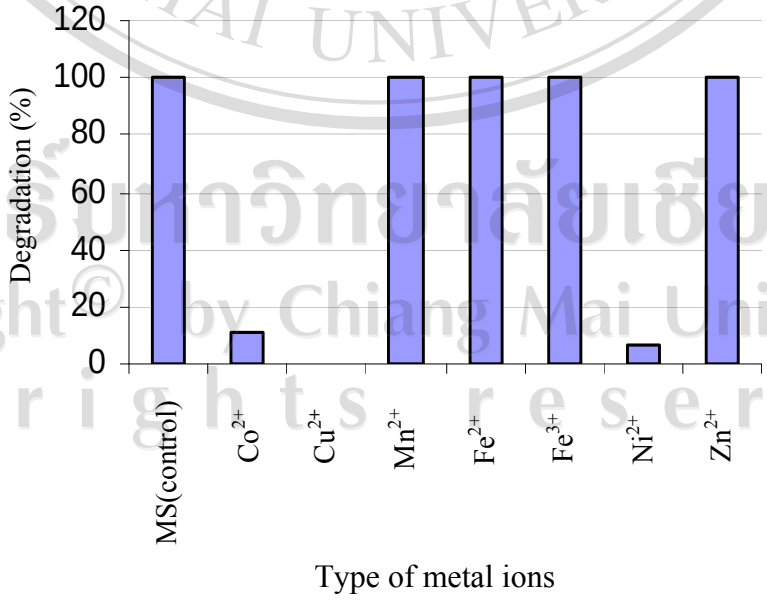


Figure 4.6 The effect of metal ions on phenol degradation by *C. tropicalis* CMU 10.

4.7 Phenol degradation by free and immobilized cells in repeated batch cultures

Free, alginate- and agar-immobilized cells of *C. tropicalis* CMU 10 were examined for degradation of phenol using different initial phenol concentrations. Phenol could be completely degraded for 4, 7 and 5 rounds of sequential batch, respectively. Free yeast cells and immobilized cells in agar beads were able to degrade phenol at an initial concentrations up to 1,750 and 2,000 mg/L within 246 and 1,578 h, respectively (Figure 4.7a,c) whereas cells immobilized in alginate beads were able to tolerate higher phenol concentration and completely mineralized phenol at the initial concentration of 2,500 mg/L within 432 h (Figure 4.7b). The absence of lag phase during the first few rounds of sequential batch experiments was not unexpected because the yeast cells were adapted and propagated in the cultivation media with phenol. The overall removal of phenol was consistently and invariably high at 100%. Further increase of the initial phenol concentration to 2,750 mg/l (Figure 4.7b) resulted in less degradation (27 % of phenol was degraded). The cessation of phenol degradation at this stage might be the results of cells death. The problem of gradual cell leakage from the immobilized beads throughout the sequential batch system was rectified by beads stabilization and resulted in the improved alginate and agar beads that were able to successfully degraded phenol for 8 and 6 rounds of sequential batch.

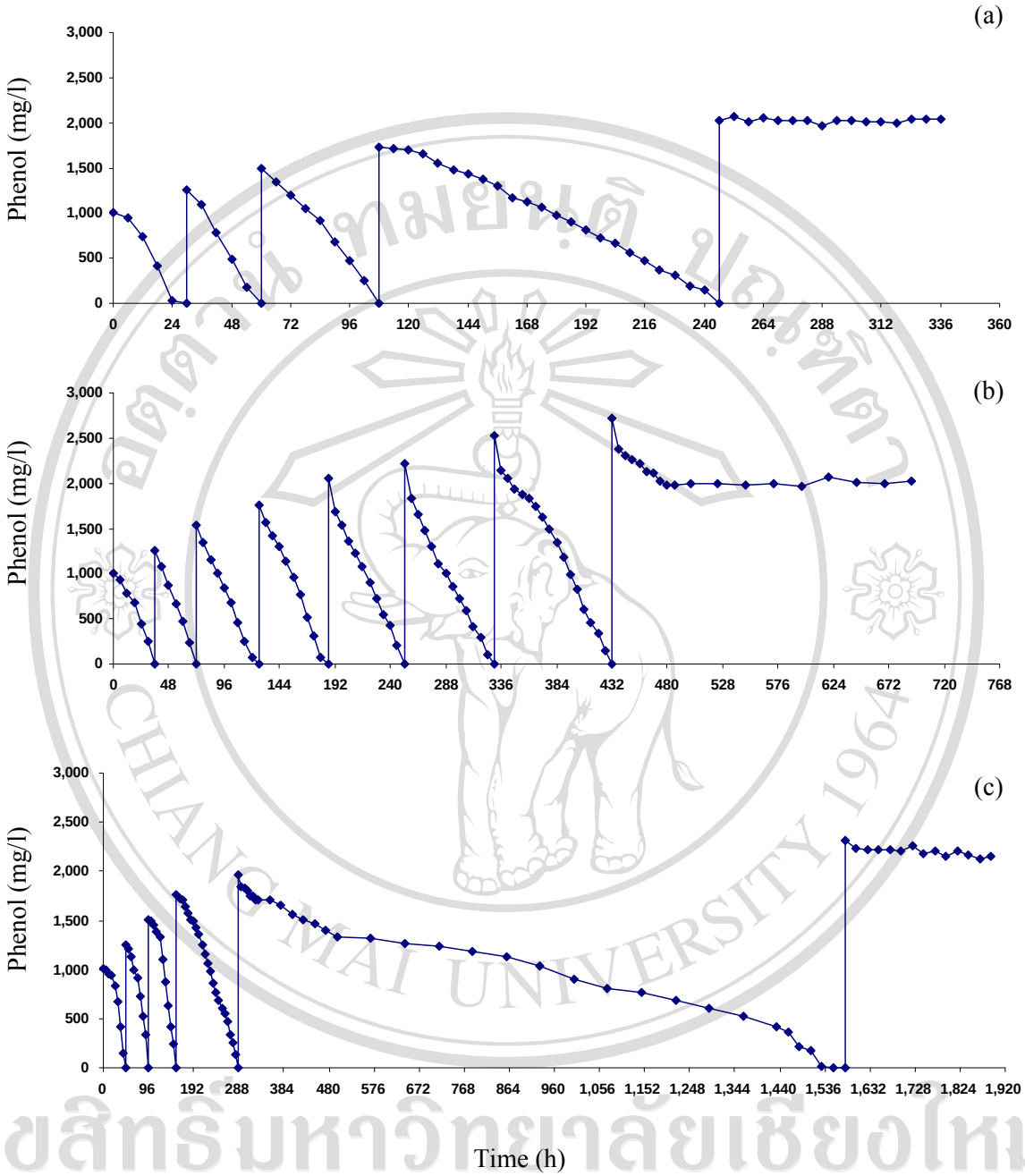


Figure 4.7 Degradation of phenol by *C. tropicalis* CMU 10 in repeated batch cultures with various level of initial phenol concentrations and cell preparation;

(a) free cells,

(b) alginate-immobilized cells,

(c) agar-immobilized cells.

The investigation of whether immobilizing agents played any role in lowering the phenol concentration through adsorption process was performed as shown in Figure 4.8. The insignificant decrease of phenol concentration was detected at the same initial concentration for both immobilization matrices. These results indicated that the decreases in phenol concentration based on adsorption were relatively small (2.7% for alginate beads and 3.3% for agar beads). Yeasts which were able to degrade up to 1,412 – 1,700 mg/l phenol were *C. tropicalis* (Bastos *et al*., 2000), *C. maltosa* (Fialova *et al*., 2004), and *Trichosporon dulcitum* (Margesin *et al*., 2005). More recently, free cells of *C. albicans* was reported for its ability to degrade phenol up to 2,260 mg/l (Tsai *et al*, 2005) while the agar-immobilized form was able to break down up to 1,560 mg/l phenol in a fluidized bed reactor (Juaxrez-Ramixrez *et al*., 2001). Some immobilized mesophilic bacteria in alginate or agar also possessed high phenol degrading activity (Zaitsev *et al*., 1995; Adav *et al*., 2007; Karigar *et al*., 2006; Santos *et al*., 2008).

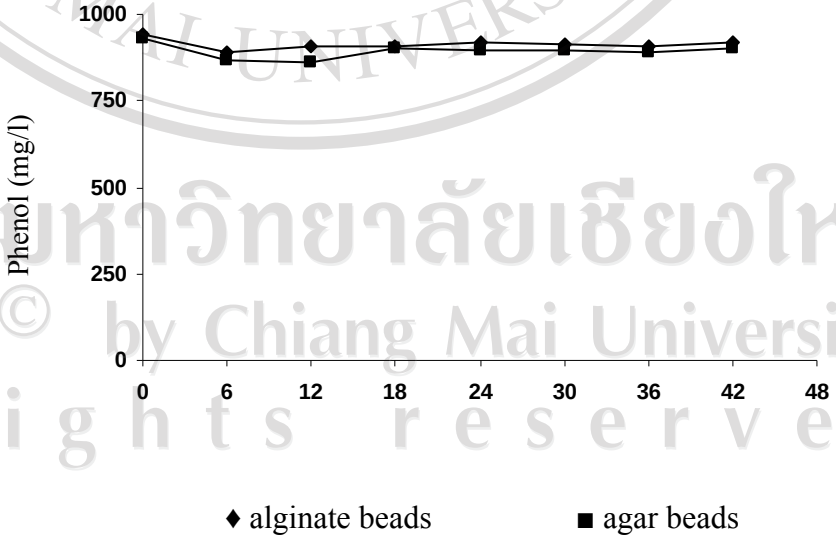


Figure 4.8 Adsorption of phenol (1,000 mg/l) on immobilizing agents.

The immobilized yeast strain in alginate- and agar-matrices was useful for developing the effective means for treating phenol-containing effluents. The reuse of these immobilized beads was also possible. As phenol had a strong inhibitory effect on the growth of free cells, the employed carrier material could act as a protective shelter against the toxicity of phenol. The improved phenol-removing efficiencies of immobilized cells was observed when phenol concentrations were greater than 1,250 mg/l. The alginate-immobilized cells could degrade higher level phenol concentration than agar-immobilized cells. The alginate-immobilized cells could remove phenol concentration up to 2,750 mg/l in comparison to that of the agar-immobilized cells which could degrade up to 2,000 mg/l phenol.

4.8 Degradation kinetics of the repeated batch systems

Phenol biodegradation by free and immobilized cells of *C. tropicalis* CMU 10 were tested in MS media containing 250 mg/l higher phenol concentration. The rates of phenol degradation as a function of corresponding initial phenol concentrations are shown in Figure 4.9 a, b and c. The maximum phenol degradation rates of free, alginate- and agar-immobilized cells were 41.6, 34.9 and 26.0 mg/l-h, respectively, all at 1,250 mg/l phenol. The results showed the performance of *C. tropicalis* CMU 10 were similar to those strains tabulated in Table 2.4. The capacity of the immobilized cells in alginate beads to degrade phenol was equally high during the first five cycles. However, a decrease in rate was observed in the sixth cycle. The phenol biodegradation with immobilized cells in agar beads was less than alginate beads. The contributing factors to this observation might be inconsistency of agar gel and a relatively high solidifying temperature that might lead to thermal damage of the cells. This was in contrast to the milder preparation condition of calcium alginate. The different level of porosity and pattern of accumulated biomass in gel matrix might generate discrepancy in diffusion limitations that interfere with cell growth (Freeman and Lilly, 1998). The alginate beads were evidently more suitable for the operation of several consecutive biodegradation batches. At phenol concentrations up to 1,250 mg/l, the higher degradation rate was achieved using free cells rather than the immobilized counterparts. The resistance of immobilized cells to phenol toxicity might be due to morphological changes and adsorption on the support material which delayed the diffusion of toxic substances into the cells (Dias *et al.*, 2001). The phenol degradation might be limited by the diffusion of phenol and/or oxygen within the gel

beads matrix. If the phenol concentration was sufficiently low, the toxic effect of substrate might not be so severe for the free cells suspension (Juaxrez-Ramixrez *et al*., 2001). However, the apparently high concentration of phenol up to 1,500 mg/l employed in the current experiment resulted in the adverse impact to the phenol degradation efficiency of free cells suspension. Due to the inhibitory and cellular lytic effects of phenol, the degradation rates were varied depending on the levels of initial phenol concentrations employed. The higher phenol concentration level resulted in the lower degradation rate. The highest phenol degradation rate of immobilized *Nocardioides* sp. NSP41 in alginate beads was 21.0 mg /l-h when 400 mg/l phenol was employed (Cho *et al*., 2000). Degradation rates of free and alginate-immobilized cells of *Aureobasidium pullulans* FE13 were 18.35 and 20.45 mg/l-h, respectively for 1,505 mg/l phenol in batch cultures (Santos *et al*., 2008).

The immobilized *C. tropicalis* CMU 10 in alginate and agar matrices were able to treat phenol-containing effluents effectively. The reuse of the immobilized cells was also possible for several rounds. However, such repeated uses would eventually lead to a decrease in phenol degradation rate (Kim *et al*., 2005).

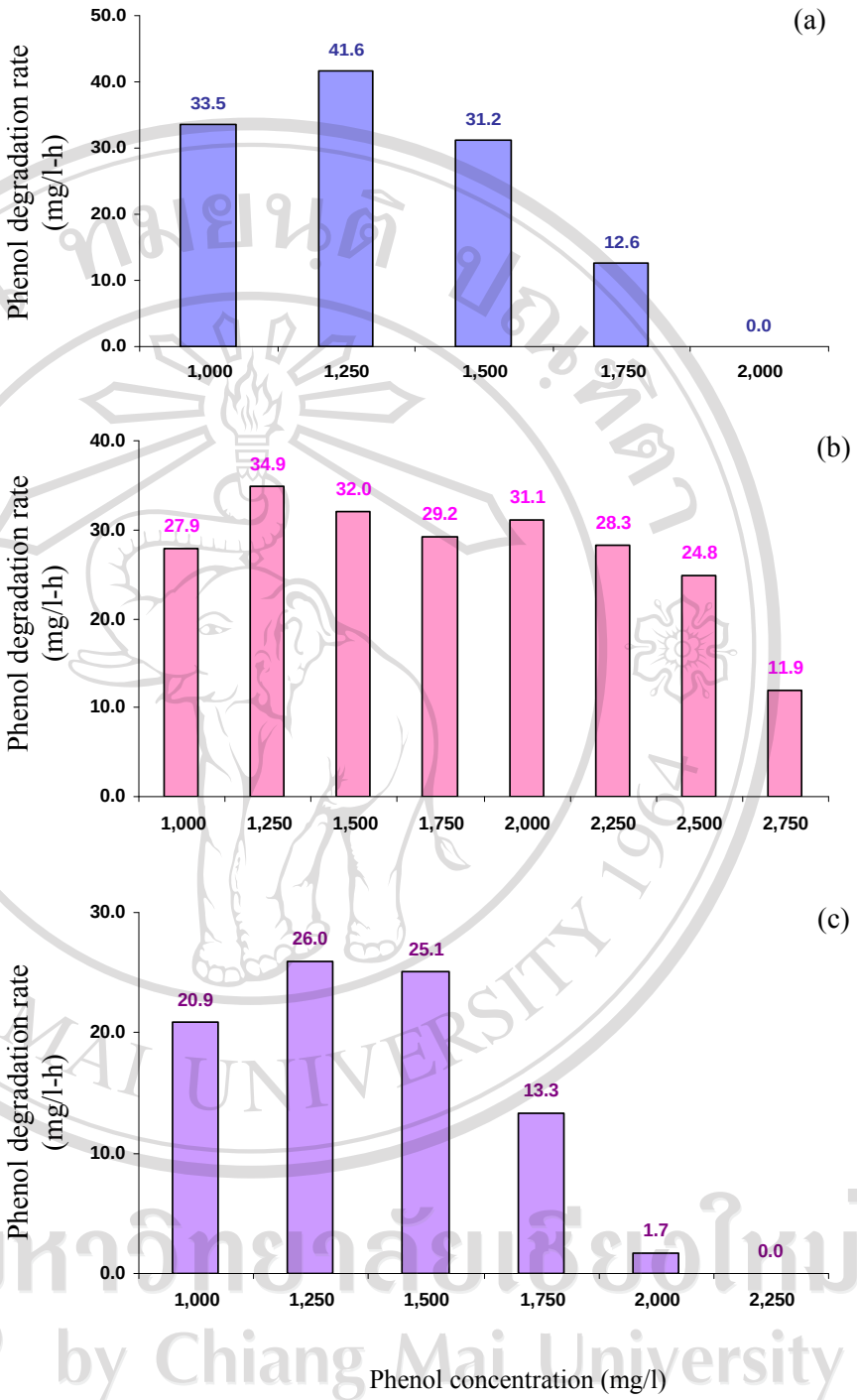


Figure 4.9 Effect of initial phenol concentrations on degradation rate by *C. tropicalis* CMU 10 in repeated batch cultures for different cells preparation;

(a) free cells,

(b) alginate-immobilized cells,

(c) agar-immobilized cells.