CHAPTER VI

RESULTS
(CONSTRUCTION OF ARTIFICIALLY STRUCTURED BACTERIAL CONSORTIUM)

6.1 Identification of bacterial isolates in the consortium contributing melanoidins decolorization

The previous experiments on the organization of bacterial consortium CONS8 in the Chapter 5 were done with of natural composition and architecture. Bacterial consortia in nature are typically formed by bacterial strains, normally heterogeneous, and often have complex structures. The organization of these consortia can be expected to have a significant influence on the function of the cells within the bacterial consortia. The ability to create new consortium with defined architecture, with different strains of bacteria, could result in a significant advance in the methods available for the optimization of bacterial decolorization and might offer a good strategy for obtaining high decolorization.

Hence, in the present experiments, the study was focused on the construction of a high potential melanoidins decolorizing bacterial consortium. The aim was to investigate the possibility of constructing artificially structured bacterial consortium for decolorization of the synthetic melanoidins-containing wastewater medium.

A prerequisite for this study was the ability to separate species on selective media to facilitate analysis. Serial subcultures of the consortium CONS8 were carried out on melanoidins-containing wastewater medium to discriminate the effective melanoidins-decolorizing species.

Subsequently, the decolorizing bacterial isolates present after 5 successive subcultures were isolated to pure culture by morphological difference. This was performed by examining growth, colony size, color, and morphology on LB medium agar plates after incubation for 24 h and confirmed by Gram staining. The different individual bacterial colonies on LB agar plates are shown in Figure 6.1.

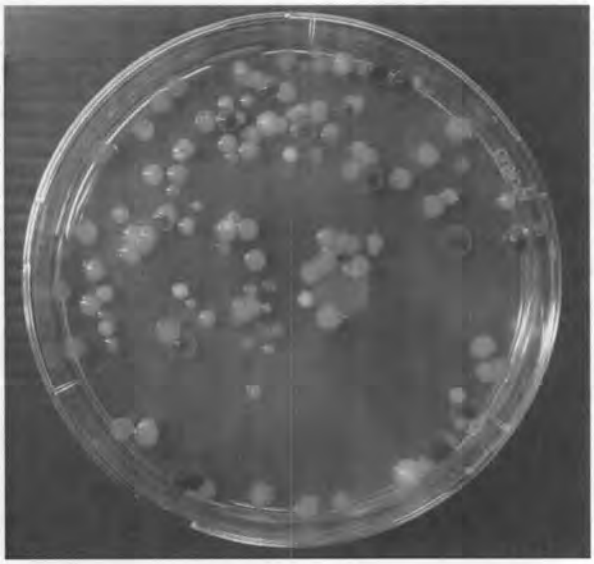

Figure 6.1 View of the various bacterial colonies on LB agar plate after 5 successive subcultures of consortium CONS8 under aerobic conditions.

The individual bacterial isolates shown in Figure 6.1 were grown in LB agar plate under aerobic conditions for 24 h at 30^{o}C. Each isolate in the consortium CONS8 was further identified by partial 16S rDNA sequence. The 16S rDNA gene PCR amplified from the isolates using 20F and 802R primers was subjected to DNA sequencing with UFUL internal primers (Nilsson et al., 2003). The sequences were then compared using NCBI BLASTN program. Pairwise alignments giving a closest match of 99% or more were chosen.

The enriched consortium CONS8 mainly comprised of *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), *Citrobacter farmeri* (T3) and unknown bacterium DQ817737 (T4). The 16S rDNA sequences of these bacteria were shown in Appendix 2.

Some of these species have been reported as molasses decolorizing bacteria. They were *Serratia mercescens*, *Citrobacter* sp and *Klebsiella* present in this bacterial consortium. All of them could decolorize colored components present in molasses wastewater (Kumar and Chandra R, 2006; Mohana et al., 2007; Petruccioli, 2000). Many researchers also reported the activity of *Serratia mercescens* on biodegradation of polycyclic aromatic hydrocarbons (PAHs) and lignin degrading activity (Rhoads et al., 1995).

6.2 Construction of artificially structured bacterial consortia

To verify whether the decolorization of melanoidins-containing wastewater is more effective by artificially constructed bacterial consortia than either consortium CONS8 or individual bacterial strains, the experiment was carried out by constructing different bacterial consortia. Factorial experimental design was applied to identify the potent melanoidins-decolorizing bacterial isolates and to determine the efficient bacterial compositions.

The four predominant bacterial members present in the consortium CONS8, *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), *Citrobacter farmeri* (T3) and unknown bacterium (DQ817737) (T4) were chosen as independent variables and the percentage of decolorization was the dependent response variable.16 different experiments were carried out according to the factorial method for 4 bacteria (T1-T4) in comparison with control (Table 6.1.).

Table 6.1 Experimental design for decolorization of the synthetic melanoidins-containing wastewater medium with the four isolates

Experiments	Bacterial composition
1	T1
2	T2
3	T3
4	T4
5	T1+T2
6	T1+T3
7	T1+T4
8	T2+T3
9	T2+T4
10	T3+T4
11	T1+T2+T3
12	T1+T2+T4
13	T1+T3+T4
14	T2+T3+T4
15	T1+T2+T3+T4
control	No bacterium

Note: T1= *Serratia mercescens*, T2= *Klebsiella oxytoca*, T3= *Citrobacter farmeri* and T4= unknown bacterium DQ817737

The summarized results of decolorization of the synthetic melanoidins-containing wastewater by individual strains and by different mixture of strains after incubation under aerobic conditions for 72 h are shown in Figure 6.2. The decolorization varied among experiments . The bacterial growth is also presented in Figures 6.3 – 6.18. They showed that the effectiveness of decolorization depends on bacterial components of the inoculum. The results indicated that the decolorization varied significantly in the range of 0 – 17.5% with the bacterial composition tested.

Individually, unknown bacterium (DQ817737) (T4), *Klebsiella oxytoca* (T1) and *Citrobacter farmeri* (T3) could decolorize melanoidins-containing wastewater up to 11.4%, 8.94% and 6.72% in 72 h, respectively, under aerobic conditions (Figure 6.2). The results show that unknown bacterium DQ817737 (T4) accounted for the

highest of decolorization of synthetic melanoidins-containing wastewater. Lowest decolorization was observed when pure culture of *Serratia mercescens* (T2) was used as inoculum (experiment 2).

Although *Serratia mercescens* (T2) was not an effective decolorizer, its presence might still play an important role in increasing color removals of various bacterial consortia (experiment 5, 8, 11, 12, 14 and 15). It seems that decolorization activities in these experiments was enhanced by the presence of *Serratia mercescens* (T2).

The experimental results also illustrated that the bacterial consortia were able to decolorize synthetic melanoidins-containing wastewater at higher level as compared to those achieved by individual isolates. Decolorization values above 15% were only observed when consortia were composed of strain T1, T2 and T4 together (experiment 12 and 15). The experimental results suggest that the bacterial composition obviously affect the decolorization.

The result in Figure 6.3 shows that the decolorization did not occur in sterile cell-free medium, suggesting the absence of abiotic decolorization.

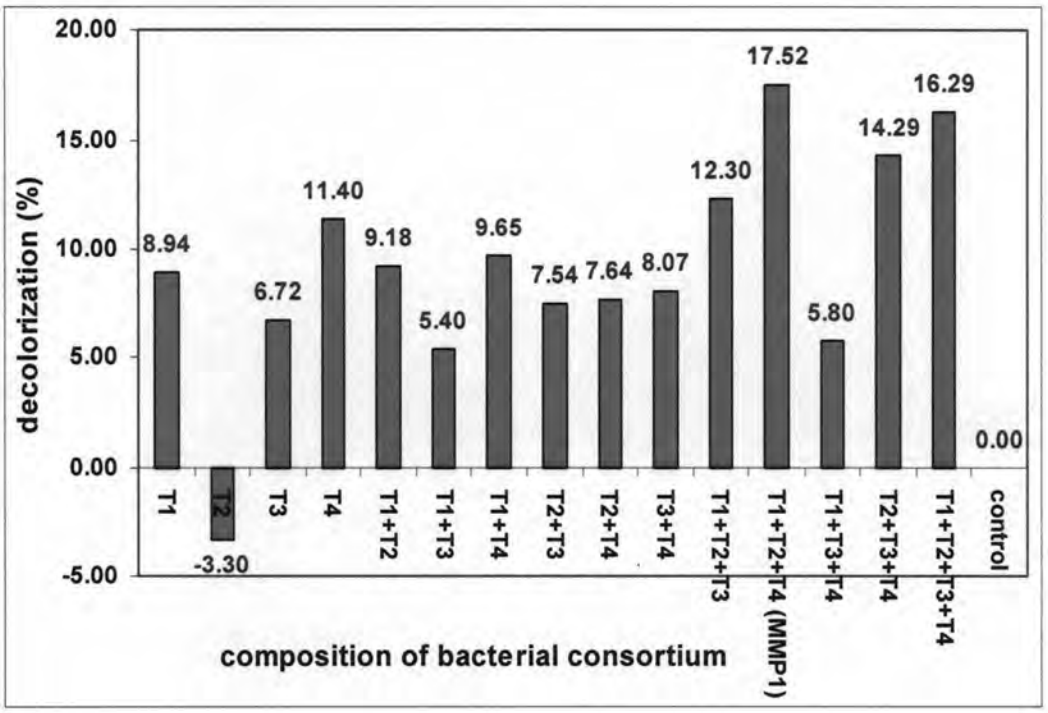

Figure 6.2 Percent decolorization of the synthetic melanoidins-containing wastewater medium containing 2% (v/v) Viandox by different bacterial consortia. Negative value indicates the increase in color; the data result of three independent experiments.

As might be expected for very simple bacterial consortia, these results suggested that *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), and unknown bacterium (DQ817737) (T4) strongly contributed to the decolorization of melanoidins in many consortia as shown in experiment 5, 8, 11, 12, 14 and 15.

As shown in Figure 6.2, a consortium MMP1 comprising of three bacteria; *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), and unknown bacterium (DQ817737) (T4) exhibited the same degree of decolorization to the mixed population with the four bacteria (experiment 15). Moreover, the degree of decolorization (17.5 %) of the melanoidins by the consortium MMP1 was almost equal to those of the bacterial consortium CONS8 with natural architectures under the same culture conditions (Figure 5.4a). Thus, bacterial consortium MMP1 appeared to be a good choice rather than consortium CONS8 in term of prolonging the high decolorization efficiency and suitable for in situ application.

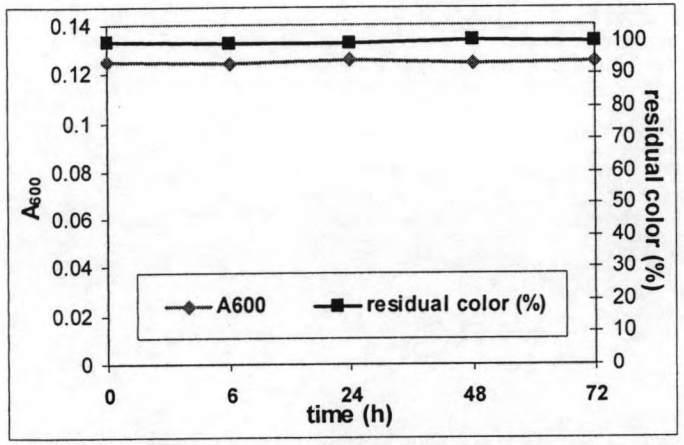

Figure 6.3 Time course of growth and decolorization of the control experiment (without bacteria)

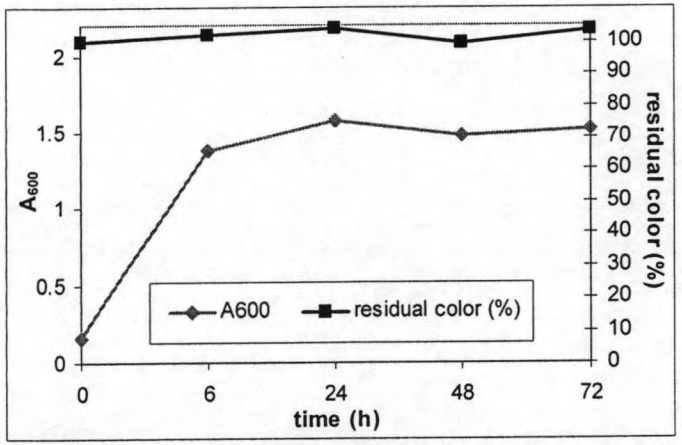

Figure 6.5 Time course of growth and decolorization of the experiment 2 (culture T2)

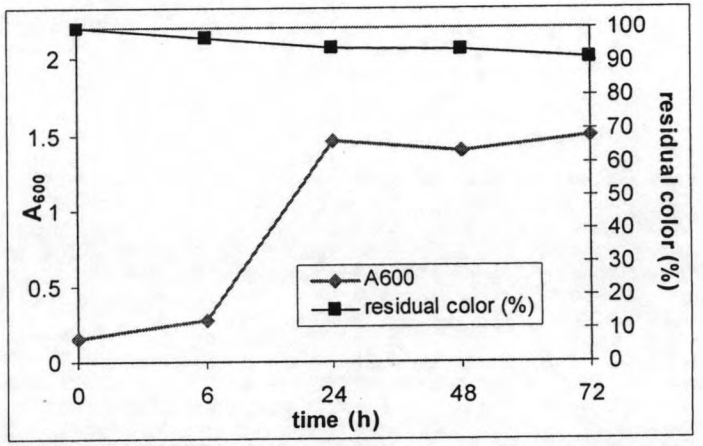

Figure 6.4 Time course of growth and decolorization of the experiment 1 (culture T1)

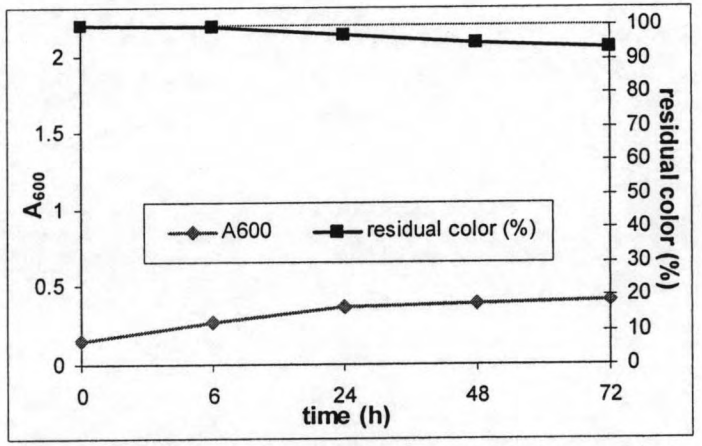

Figure 6.6 Time course of growth and decolorization of the experiment 3 (culture T3)

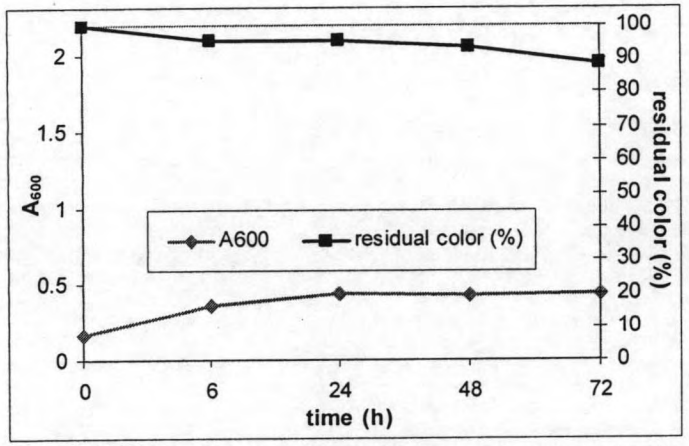

Figure 6.7 Time course of growth and decolorization of the experiment 4 (culture T4)

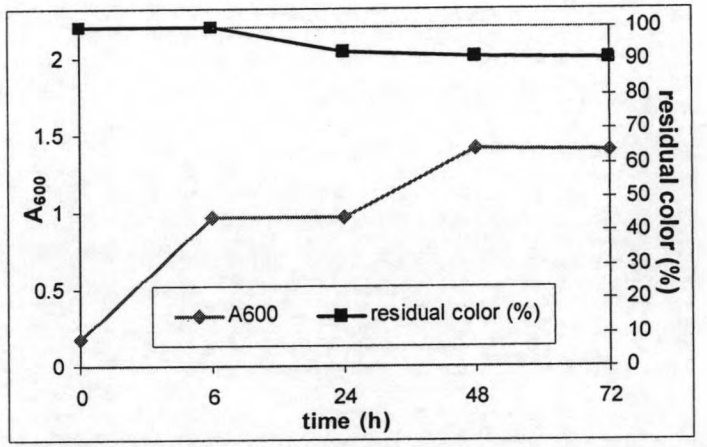

Figure 6.8 Time course of growth and decolorization of the experiment 5 (co-culture T1+T2)

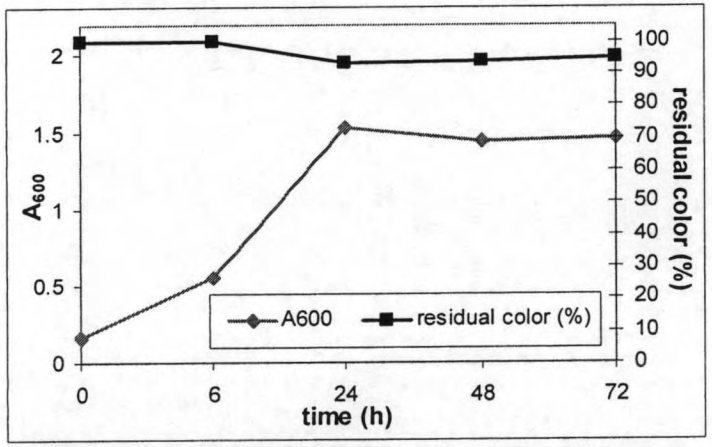

Figure 6.9 Time course of growth and decolorization of the experiment 6 (co-culture T1+T3)

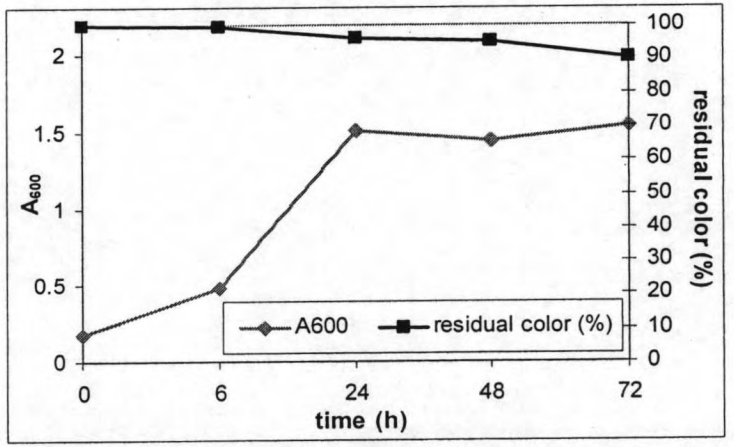

Figure 6.10 Time course of growth and decolorization of the experiment 7 (co-culture T1+T4)

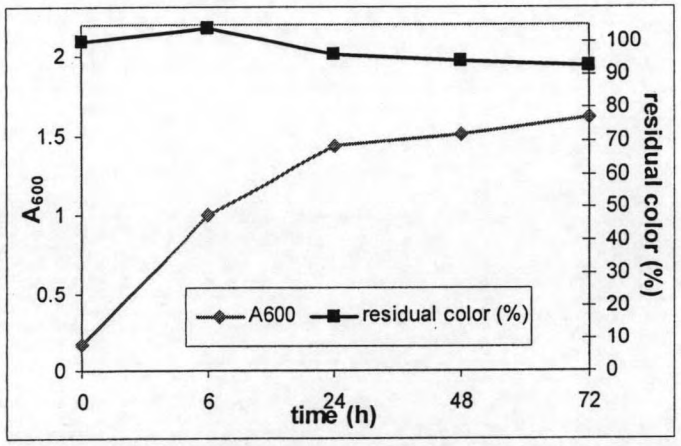

Figure 6.11 Time course of growth and decolorization of the experiment 8 (co-culture T2+T3)

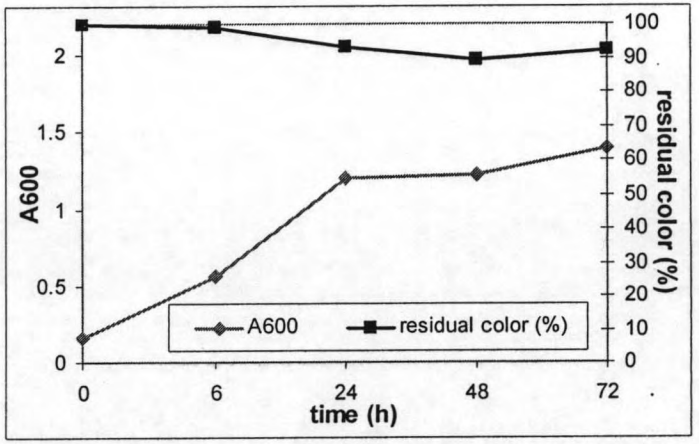

Figure 6.12 Time course of growth and decolorization of the experiment 9 (co-culture T2+T4)

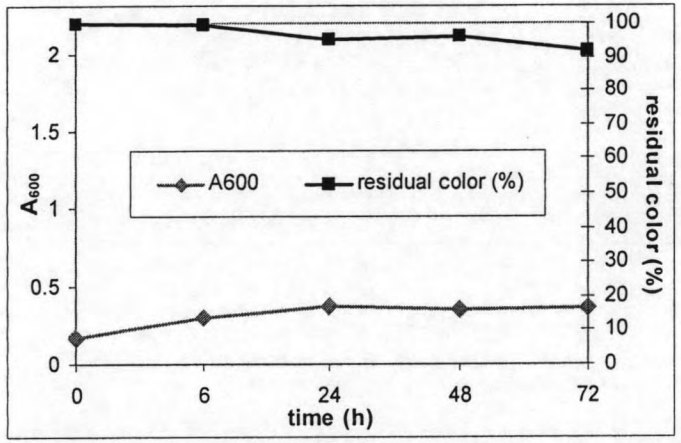

Figure 6.13 Time course of growth and decolorization of the experiment 10 (co-culture T3+T4)

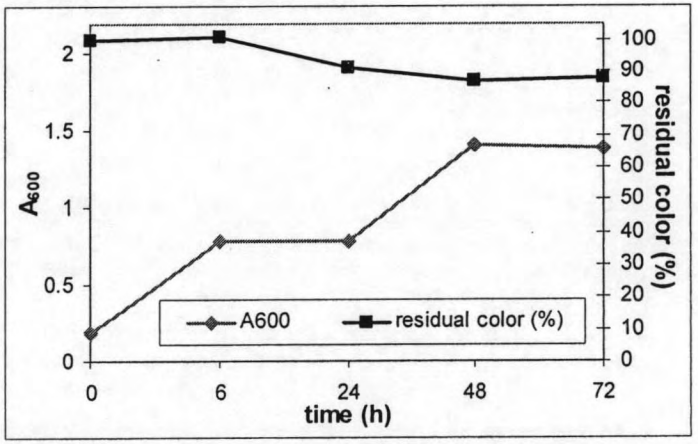

Figure 6.14 Time course of growth and decolorization of the experiment 11 (co-culture T1+T2+T3)

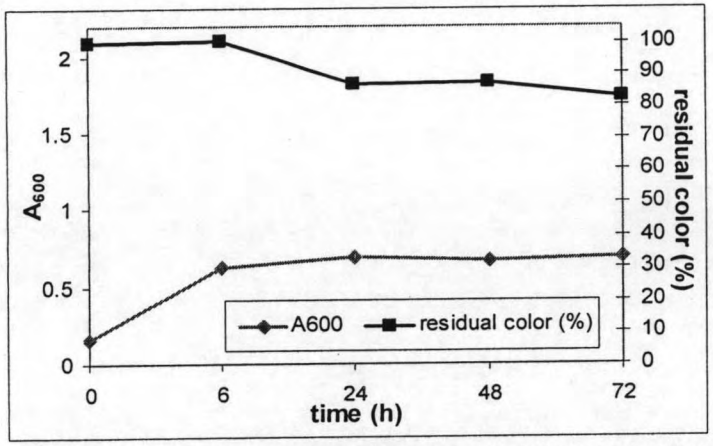

Figure 6.15 Time course of growth and decolorization of the experiment 12 (co-culture T1+T2+T4)

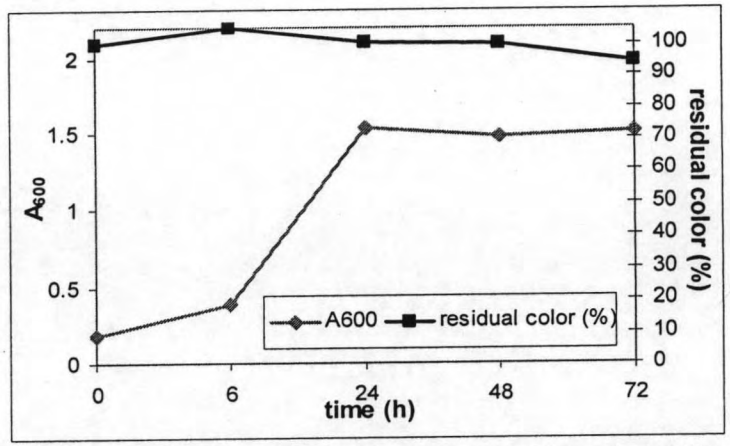

Figure 6.16 Time course of growth and decolorization of the experiment 13 (co-culture T1+T3+T4)

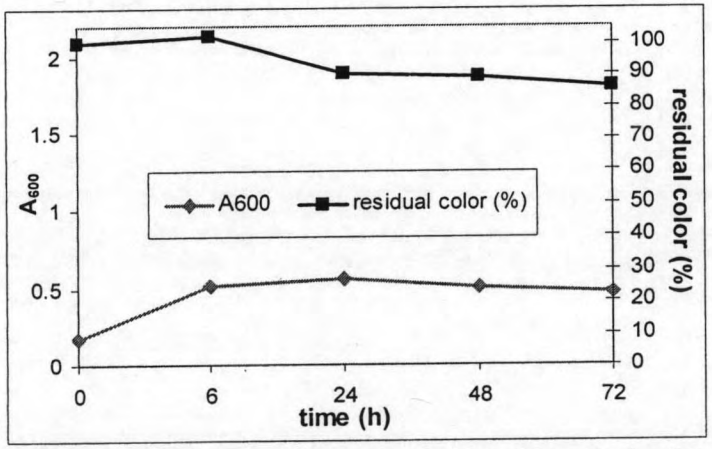

Figure 6.17 Time course of growth and decolorization of the experiment 14 (co-culture T2+T3+T4)

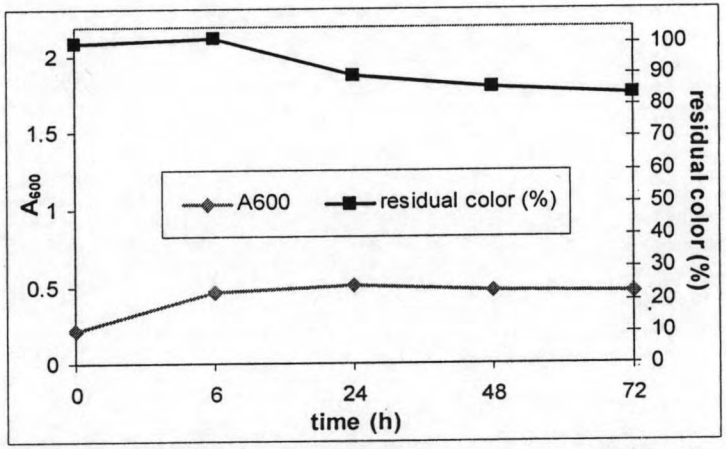

Figure 6.18 Time course of growth and decolorization of the experiment 15 (co-culture T1+T2+T3+T4)

In this experiment, the bacterial consortium namely MMP1, comprising of *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), and unknown bacterium DQ817737 (T4), was tested in further study due to its highest decolorization level 17.52%. Since the melanoidin decolorization process is enzyme dependent, the consortium showed synergistic effect and adapted metabolism to produce nontoxic metabolites. Furthermore, bacterial decolorization may require a mixed culture to decolorize molasses wastewater through combined metabolic mode of individual bacterial strain. Hence, the higher decolorization efficiency of consortium MMP1 may be due to the enhanced effect of coordinated metabolic interactions on melanoidins decolorization (Manjinder et al., 2005; Sarayu et al., 2006; ; Sarayu et al., 2008).

A similar pattern was also observed on the decolorization of bacterial consortium DMC, comprising of *Pseudomonas aeruginosa* PAO1, *Stenotrophomonas maltophila* and *Proteus mirabilis*, which achieved its maximum molasses decolorization activity 67% and 51% COD reduction within 72 h in the presence of 0.5% glucose (Mohana et al., 2007).

Similarly, Nigam et al. (1996) reported that although the individual bacterial isolates present in the consortium-PDW were unable to decolorize any dyes, however, the mixed bacterial culture-PDW was able to decolorize various textile dyes with a rather slow decolorization rate.

Senan and Abraham (2004) also developed a consortium of three bacteria to degrade a mixture of the dyes by co-metabolism and observed that the consortium could decolorize efficiently all the three dyes tested.

Hence, mixed bacterial culture seems to be more promising for molasses wastewater decolorization. This is due to the synergism present in mixed communities, as the catabolic activities of bacteria in the consortium complement each other.

6.3 Optimum decolorization condition of constructed bacterial consortium MMP1

To elucidate the cooperative action of mixed populations in the decolorization of melanoidins-containing wastewater, the artificial bacterial consortium MMP1 composed of three bacteria was used. Experiments were repeated to validate the optimum condition of bacterial strains in consortium MMP1. The decolorization efficiency of bacterial consortium MMP1 was investigated under the optimum condition. Precultures of *Serratia mercescens*, *Klebsiella oxytoca*, and unknown bacterium (DQ817737) were prepared in the synthetic melanoidins-containing wastewater medium. Decolorization of melanoidins by bacterial consortium MMP1

comprised of these three strains was examined in synthetic melanoidins-containing wastewater medium containing a Viandox sauce (2%, v/v) as a color substrate with the initial pH 4 at 30°C under aerobic conditions for 72 h.

Figure 6.19 shows a typical culture profile of the constructed bacterial consortium MMP1. The plots of percentage decolorization (Figure 6.19) revealed the profile of decolorization with respect to changes in bacterial biomass, total nitrogen concentration and pH. These culture conditions resulted in approximately of 22% decolorization of Viandox sauce within 72 h. Further incubation did not enhance the decolorization. The total nitrogen decreased from 390 mg/L at the beginning of this experiment to 290 mg/L after incubation for 10 h and remained unchanged until 72h. The pH of cultured medium showed no significantly change during the overtime (Figure 6.19).

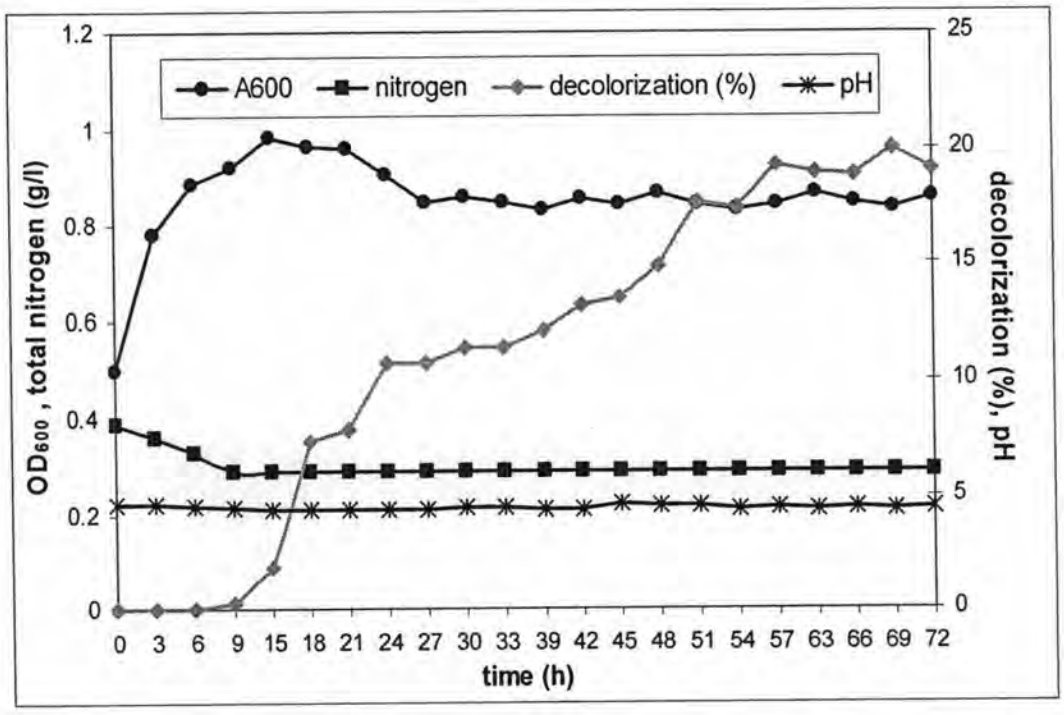

Figure 6.19 Growth and decolorization of melanoidins-containing wastewater by the constructed bacterial consortium MMP1 under optimal conditions.

In this chapter, the results indicated that the bacterial consortium MMP1 was able to decolorize synthetic melanoidins-containing wastewater. The optimum combination of the bacterial members (T1, T2, and T4) in the bacterial consortium MMP1 was confirmed through experiments and hence this constructed bacterial consortium holds a potential for the treatment of melanoidins-containing wastewaters. Application of the artificially constructed bacterial consortium MMP1, comprising *Serratia mercescens*, *Klebsiella oxytoca*, and unknown bacterium (DQ817737) to decolorization of melanoidins-containing wastewater seems to be a

pragmatic approach. Thus, further studies were carried out by using the artificial constructed bacterial consortium MMP1.

6.4 Optimum aeration condition of constructed bacterial consortium MMP1 in bioreactor

Aeration basically serves the purpose of providing the air required for biodegradation and keeping the biomass dispersed throughout the reactor. To investigate the effect of aeration on decolorization of synthetic melanoidins-containing wastewater medium by bacterial consortium MMP1, the experiment was carried out in a 3L laboratory-scale completely stirred tank reactor contained 2L of solution (BioFlo® 110 Fermentor & Bioreactor; New Brunswick Scientific Co., Inc. USA). The experimental set up used in this study is shown in Figure 6.20. The air was supplied through the reactor liquid phase using an air sparger at the bottom. DO concentration in the reactor was measured by a CO_2/O_2 analyzer (Xentra 4100 Gas Purify Analyser; Servomex S.A. France) (Figure 6.21). The oxygen concentration was varied at 0, 0.1, 0.2, and 0.4 vvm during the start-up by subsequent adjustment of oxygen loading by gas flowmeter. The calculation of K_La values at various aeration rates was shown in Appendix 3. The oxygen transfer coefficient (K_La) in the synthetic melanoidins-containing wastewater at various aeration rates was shown in Figure 6.22. Full mixing within the reactor was achieved with one set of mechanical stirrer, which was at the bottom of the liquid phase. The agitation speed was set at 150 rpm. Reactor was equipped with heat jackets in order to maintain an operational temperature of $30^{o}C$. The influence of DO on melanoidin decolorization by the bacterial consortium in bioreactor was investigated after the seed bacterial inoculation. Samples were taken for measurements of optical density at 600 and 475 nm.

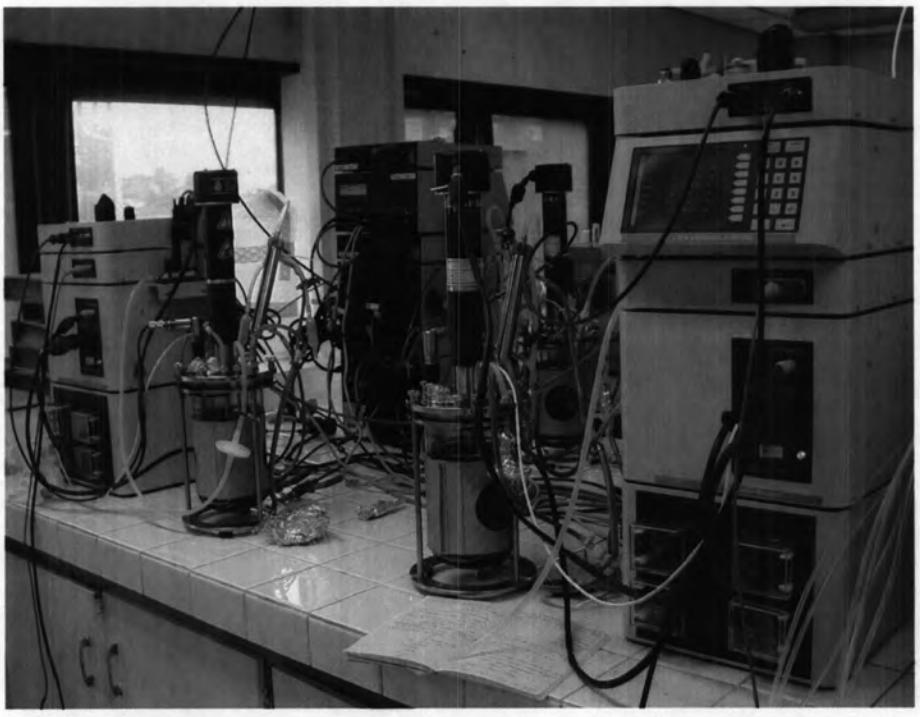

Figure 6.20 Laboratory-scale suspended cell bioreactor. (BioFlo® 110 Fermentor & Bioreactor; New Brunswick Scientific Co., Inc. USA)

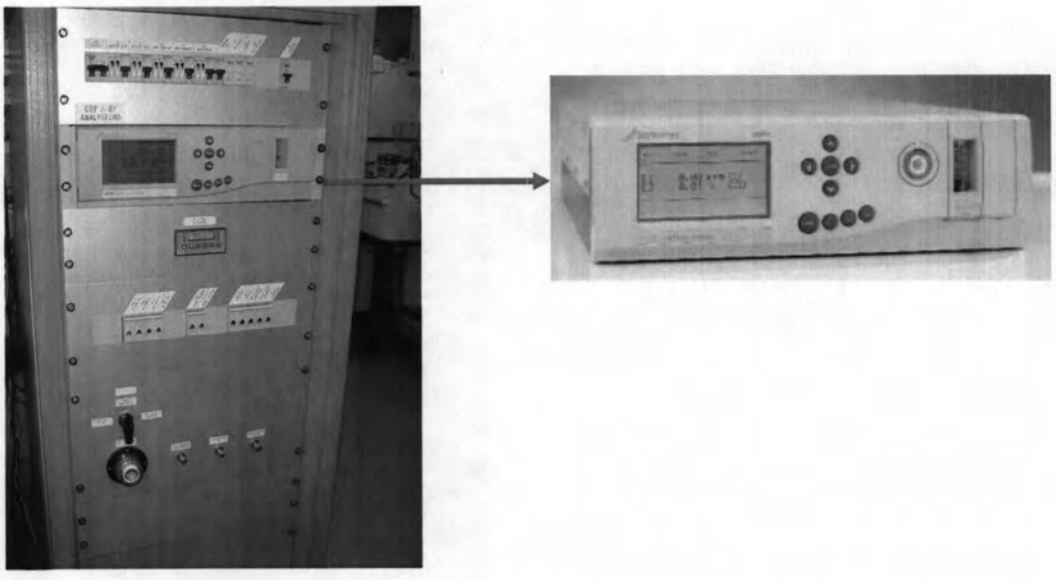

Figure 6.21 CO_2/O_2 analyzer used in this study. (Xentra 4100 Gas Purify Analyser; Servomex S.A. France)

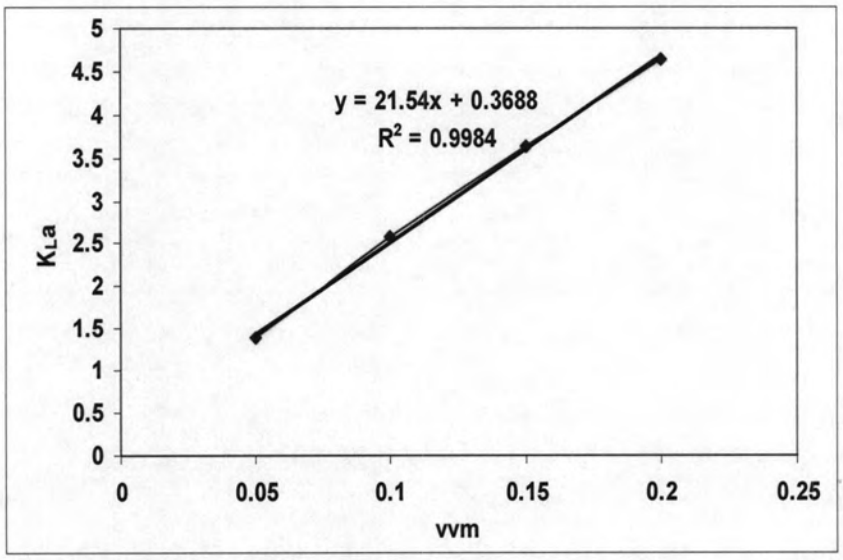

Figure 6.22 Correlation between oxygen transfer coefficient (K_La) at various aeration rates in the synthetic melanoidins-containing wastewater

The bacterial consortium could grow and showed decolorization activity around 18.92%, 19.27%, 16.94% and 8.31%, when cultivation at the aeration rate of 0 vvm (K_La=0.3688 h^{-1}), 0.1 vvm (K_La= 2.5836 h^{-1}), 0.2 vvm (K_La= 4.6343 h^{-1}), and 0.4 vvm (K_La= 8.9848 h^{-1}), respectively (Figure 6.23, 6.24, 6.25 and 6.26).

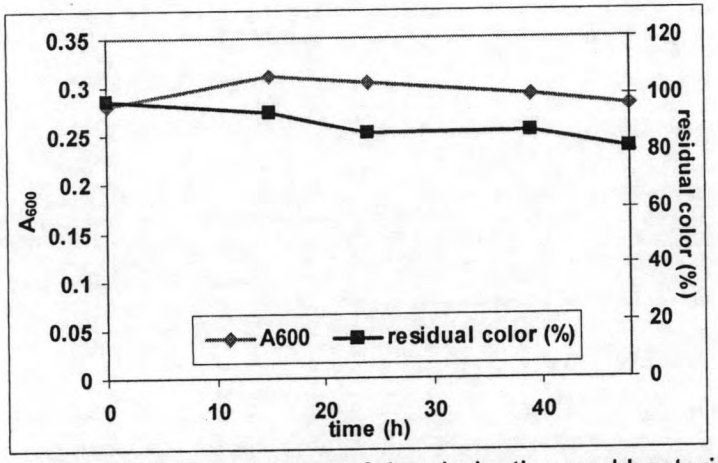

Figure 6.23 Time course of decolorization and bacterial growth using an aeration rate of 0 vvm (K_La=0.3688 h^{-1}).

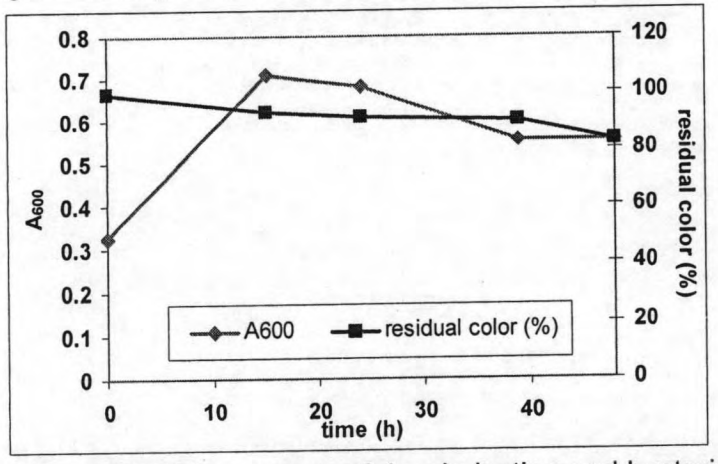

Figure 6.25 Time course of decolorization and bacterial growth using an aeration rate of 0.2 vvm (K_La= 4.6343 h^{-1}).

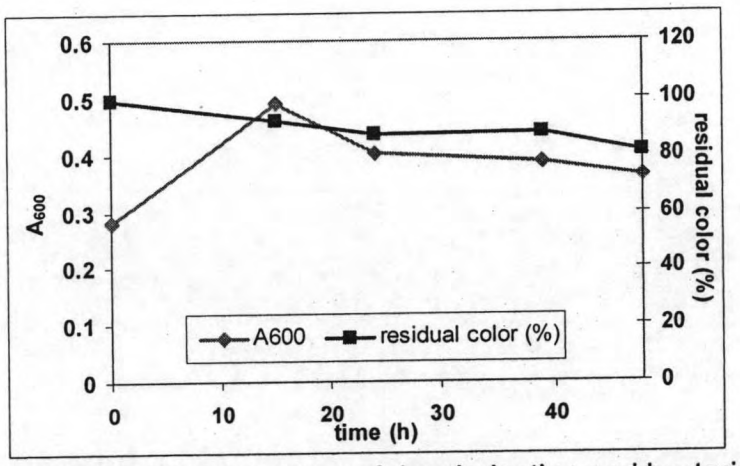

Figure 6.24 Time course of decolorization and bacterial growth using an aeration rate of 0.1 vvm (K_La= 2.5836 h^{-1}).

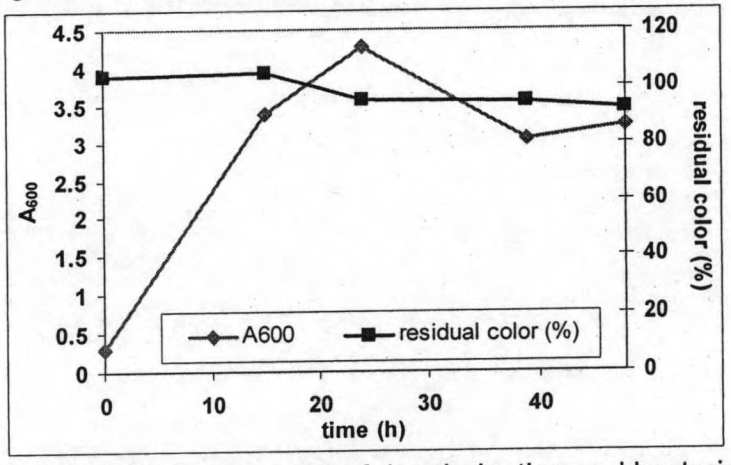

Figure 6.26 Time course of decolorization and bacterial growth using an aeration rate of 0.4 vvm (K_La= 8.9848 h^{-1}).

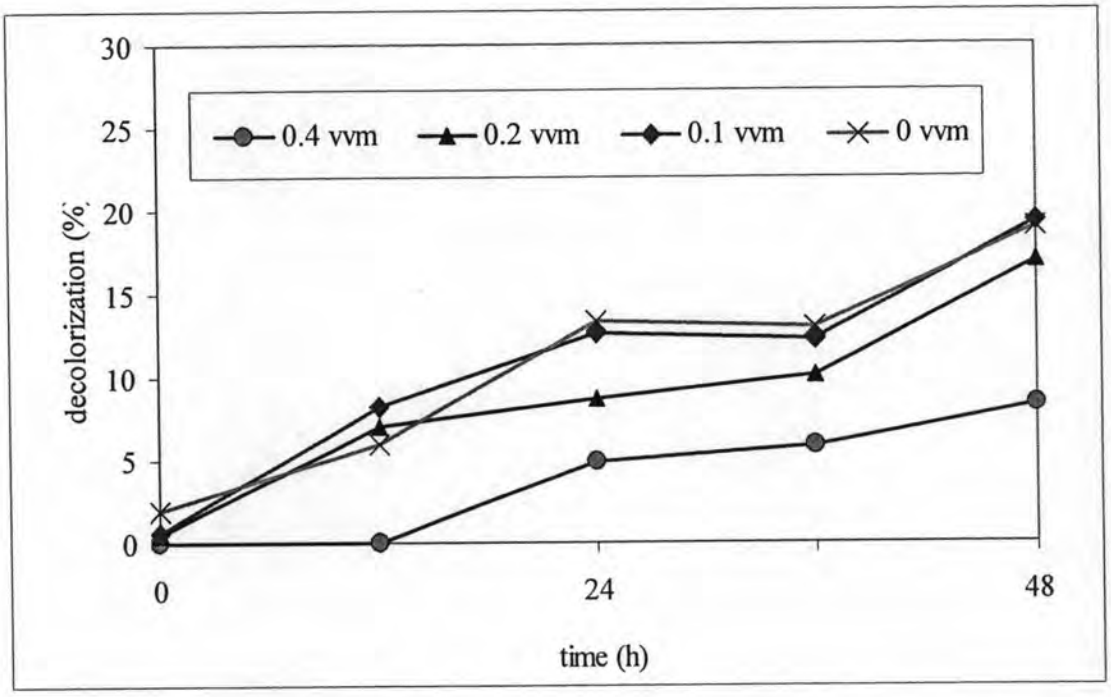

Figure 6.27 The effect of aeration rate on decolorization of synthetic melanoidins-containing wastewater containing 2% (v/v) Viandox as a color substance.

Figure 6.27 showed that the consortium could decolorize synthetic melanoidins-containing wastewater medium at aeration rate of K_La=0.3688 h^{-1} (0 vvm), K_La= 2.5836 h^{-1} (0.1 vvm), K_La= 4.6343 h^{-1} (0.2 vvm), and K_La= 8.9848 h^{-1} (0.4 vvm) up to 18.92%, 19.32%, 16.94%, and 8.31% within 48 h, respectively. Further increase in the aeration rate, instead of improving the decolorization, inhibited it. Finally, the best condition for decolorization seemed to be on closed to anaerobic condition. Then the aeration rate for further melanoidins decolorization was chosen as K_La= 2.5836 h^{-1} (0.1 vvm).

The explanation of this behavior may be found in the fact that the microorganisms that have been shown to degrade melanoidins are not exactly suitable for treating melanoidins-containing effluent from distilleries. However, the results presented in this study showed that color removal under low aeration conditions relatively higher than the highly aerobic condition. Hence, the decolorization mechanisms of melanoidins-containing wastewater by bacterial consortium MMP1 in this study might be due to metabolism of bacterial cell under facultative and anaerobic conditions such as fermentation and anaerobic respiration (Ames et al., 1999; Sirianuntapiboon and Prasertsong, 2008).