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MICROAEROPHILIC AND ANAEROBIC DECOLORIZATION OF AZO DYE IN RICH AND MINIMAL MEDIA BY *CITROBACTER* SP. STRAIN L17

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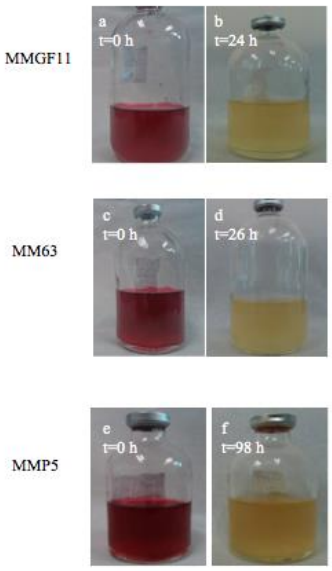
Graphical abstract



Abstract

This study aimed to investigate the ability of *Citrobacter* sp. strain L17 to decolourise azo dye in a rich medium (MP5) and three different minimal media (MMP5, MMGF11 and MM63) under microaerophilic and anaerobic conditions. Amaranth was used as the model dye in this investigation. Under microaerophilic condition, reactions were carried out at two different temperatures; 37°C and 45°C, whereas experiments with anaerobic condition were conducted only at 37°C. Results showed that, under microaerophilic condition, full decolourisation of Amaranth was achieved in all media tested at 37°C. However, at 45°C, complete decolourisation was observed only in MP5, MM63 and MMGF11 but no obvious decolourisation occurred in MMP5. On the other hand, complete decolourisation was observed in all media tested under anaerobic condition at 37°C, with the fastest decolourisation in MP5 (rich medium containing glucose and nutrient broth) and the slowest in MMP5. It was found that an inorganic buffer containing glucose at lower concentration was sufficient to achieve complete decolourisation under anaerobic condition. This finding is essential to identify a suitable medium for future study on biogas production by dye-degrading bacteria, which mostly requires anaerobic conditions.

Keywords: Decolourisation, wastewater treatment, azo dye, bacteria, microaerophilic, anaerobic

Abstrak

Kajian ini bertujuan untuk menyiasat keupayaan *Citrobacter* sp. L17 untuk menyahwarnakan pewarna azo dalam media yang kaya (MP5) dan tiga media ringkas yang berbeza (MMP5, MMGF11 dan MM63) di dalam keadaan mikroaerofilik dan anaerobik. Amaranth telah digunakan sebagai model pewarna dalam kajian ini. Di dalam keadaan mikroaerofilik, tindak balas telah dijalankan di dua suhu yang berbeza; 37°C dan 45°C, manakala eksperimen dengan keadaan anaerobik telah dijalankan hanya pada 37°C. Hasil kajian menunjukkan bahawa, di dalam keadaan mikroaerofilik, penyahwarnaan penuh Amaranth telah dicapai dalam semua media diuji pada 37°C. Walau bagaimanapun, pada 45°C, penyahwarnaan lengkap didapati di dalam MP5, MM63 dan MMGF11 tetapi tiada penyahwarnaan jelas berlaku di dalam MMP5. Sebaliknya, penyahwarnaan lengkap dapat diperhatikan dalam semua media diuji di bawah keadaan anaerobik pada 37°C, dengan penyahwarnaan terpantas di dalam MP5 (media kaya yang mengandungi glukosa dan kaldu nutrien) dan paling perlahan di dalam MMP5. Kami dapati bahawa larutan penampan tak organik yang mengandungi glukosa pada kepekatan yang rendah adalah mencukupi untuk mencapai penyahwarnaan lengkap di bawah keadaan anaerobik. Penemuan ini adalah penting

untuk mengenal pasti media yang sesuai untuk kajian pengeluaran biogas oleh bakteria penyahwarna pada masa hadapan, yang kebanyakannya memerlukan keadaan anaerobik.

Kata kunci: Penyahwarnaan, rawatan air sisa, pewarna azo, bakteria, mikroaerofilik, anaerobik

1.0 INTRODUCTION

The environmental problems created by the textile industry have received increased attention for decades, because this industry is one of the largest generators of contaminated effluents [1]. The textile industry produces highly toxic and complex wastewater containing dye, surfactants and other textile additives [2]. As the textile industry grows, more dye-containing effluent is generated and due to the highly coloured nature of the dyes, pretreatment of the contaminated wastewater is required before releasing into the water bodies. Hence, an efficient and effective treatment method to handle this large amount of waste is required. Biological treatments have been reported as one of the promising methods. There are many microorganisms such as fungi [3], bacteria [4], and cyanobacteria [5] that can be used for this purpose.

Azo dyes are a type of dye that have azo bond in their structures (-N=N-). The colour of azo dyes is ascribed to the azo-bond, the associated auxochromes and also the aromatic hydrocarbons system [6]. If the dyes are broken down anaerobically, aromatic amines are generated, which are very toxic and carcinogenic [7]. Many techniques have recently been developed towards finding an efficient method for the treatment of dye-containing wastewater, with bacterial decolourisation among the widely reported ones. This is mainly due to the low cost involved and the increasing efficiency of bacterial decolourisation, which are comparable to that of other techniques. Gastrointestinal bacteria, including those from the *Enterobacteriaceae* family, have been studied extensively for this purpose [4, 8, 9]. Moutaouakkil and co-workers reported on the decolourisation of methyl red by *E. agglomerans* in 6 hour in fluidised bed bioreactor [10]. Khalid *et al.* (2009) reported on the degradation of 4-nitroaniline (a common product of azo dye degradation) by a mixed culture from sewage sludge [9], while An *et al.* (2002) reported on the decolourisation of azo dyes by *Citrobacter* sp. isolated from textile effluent treatment facility [4].

Nowadays, research has advanced towards further optimising the wastewater treatment process, to include simultaneous energy generation. A strain of *Proteus hauseri* was shown to decolourise Reactive Blue 160 in a single chamber microbial fuel cell (MFC), with simultaneous electricity generation [11]. Fernando *et al*. (2014) recently reported on the complete degradation of Acid Orange 7 in an integrated microbial fuel cell-bioreactor system [12]. An acclimatised mixed microbial culture from an operational microbial fuel cell for dye treatment was used as inoculum [12]. In another study, the ability of a dual-species consortium of *Pseudomonas putida* and *Shewanella oneidensis* for Congo Red degradation and electricity generation was investigated [13]. The consortium was shown to generate a higher current density when compared to a single-species culture. The system was able to generate bio-electricity during the dye degradation process. To the best of our knowledge, simultaneous azo dye degradation-biogas generation is not well studied. The only report so far is on the anaerobic reduction of Methyl Orange with simultaneous hydrogen production by the facultative anaerobic bacterium *Klebsiella oxytoca* GS-4-08 [14]. Thus, this research aimed to investigate the ability of *Citrobacter* sp. strain L17 to degrade azo dye under microaerophilic and anaerobic conditions, and to identify the necessary media composition required for decolourisation. The findings will later be used in biogas production studies, which require anaerobic condition.

Citrobacter sp. L17 was originally isolated from a sewage treatment facility. The genus was identified using the 16S rRNA technique. The ability of various gastrointestinal bacterial strains isolated from the same source to degrade azo dye(s) has been previously reported [15]. Hence, this paper describes the study of Amaranth degradation by *Citrobacter* sp. L17 under both microaerophilic and anaerobic condition, in rich and several minimal media.

Table 1 The components of the rich and minimal media used in this study, and summary of decolorization results

Media	Components	References	Time for maximum decolorization at 37°C (hrs, approx.)	Time for maximum decolorization at 45°C (hrs, approx.)
Inoculum: P5	K_2HPO_4 (35.3 g L^{-1}) KH_2PO_4 (20.9 g L^{-1}) NH_4Cl (2 g L^{-1}) Glucose (10 g L^{-1}) Nutrient broth (20 g L^{-1}) Trace elements (0.5% w/v)	[15]	Not tested	Not tested
Rich medium: MP5	K_2HPO_4 (35.3 g L^{-1}) KH_2PO_4 (20.9 g L^{-1}) NH_4Cl (2 g L^{-1}) Glucose (2.5 g L^{-1}) Nutrient broth (5 g L^{-1}) Trace elements (0.5% w/v)	Modified from [15]	18 (microaerophilic) 17 (anaerobic)	136 (microaerophilic) Not tested (anaerobic)
Minimal medium: MMP5	K_2HPO_4 (35.3 g L^{-1}) KH_2PO_4 (20.9 g L^{-1}) NH_4Cl (2 g L^{-1}) Trace elements (0.5% w/v)	This study	130 (microaerophilic) 98 (anaerobic)	No decolorization (microaerophilic) Not tested (anaerobic)
Minimal medium: MM63	K_2HPO_4 (35.3 g L^{-1}) KH_2PO_4 (20.9 g L^{-1}) NH_4Cl (33 mM) $MgCl_2$ (1 mM) Glucose (0.2% w/v) Thiamine (0.3 mM) Trace elements (1% w/v)	[16]	28 (microaerophilic) 26 (anaerobic)	215 (microaerophilic) Not tested (anaerobic)
Minimal medium: MMGF11	K_2HPO_4 (35.3 g L^{-1}) KH_2PO_4 (20.9 g L^{-1}) $(NH_4)_2SO_4$ (0.1 g L^{-1}) Glucose (0.1 g L^{-1})	This study	26 (microaerophilic) 24 (anaerobic)	30 (microaerophilic) Not tested (anaerobic)

2.0 METHODOLOGY

2.1 Microorganism and Inoculum Preparation

Strain L17, from glycerol stock maintained at the Faculty of Biosciences and Medical Engineering, UTM, was cultured on nutrient agar plates at 37°C overnight. Then, a single colony was inoculated into P5 medium (Table 1) [15] at 37°C with shaking at 200 rpm overnight. The culture (10% (v/v)) was then used as starter culture for further experiments.

2.2 Decolourisation of Amaranth by Strain L17

Amaranth (C. I. Acid Red 27) (Sigma Aldrich) azo dye was used as model dye. The final dye concentration of 0.1 g L^{-1}, from a stock solution prepared in water, was used in all media [15].

2.3 Decolourisation under Microaerophilic Condition

An overnight starter culture (3 mL) grown in P5 medium was inoculated into 27 mL of fresh sterile minimal and rich media (Table 1) containing Amaranth in a 30 mL universal bottles, and capped tightly. Then, the bottles were incubated at both 37°C and 45°C with sampling performed every few hours. The extent of decolourisation was determined by centrifuging the culture and the supernatant was analysed by UV-vis spectrophotometer (at Amaranth λ_{max} of 521 nm).

2.4 Decolourisation under Anaerobic Condition

Fresh media (25 mL) was first transferred to 50 mL serum bottles and sparged with nitrogen gas. The volume of media used allows gas accumulation in the headspace of the serum bottle. After sparging, the bottles were sealed with septum and metal cap, before being sterilised by autoclaving at 121°C, for 15 minute at 15 psi. Inoculum (10%(v/v)) grown under

aerobic condition was introduced by syringe, followed by incubation at 37°C until full decolourisation was observed. Full decolourisation was estimated from the complete loss of the colour of Amaranth via direct observation.

3.0 RESULTS AND DISCUSSION

3.1 Decolouridation of Amaranth under Microaerophilic Condition in Rich Medium

Decolourisation of Amaranth under microaerophilic condition in rich media (MP5) were conducted at 37°C and 45°C, and the full results are summarised in Table 1. MP5 has glucose and nutrient broth, in addition to the buffering components and trace elements. A complete decolourisation of Amaranth was observed after 18 hours of incubation at 37°C. In contrast to this, full decolourisation in the vessels incubated at 45°C, was achieved only after 136 hours (Figure 1). As a comparison, Amaranth has been reported to be decolourised under microaerophilic condition by *Enterococcus faecalis* within 3 hours at 27-28°C [17]. Hong *et al.* (2007) reported that anaerobic decolourisation of Amaranth by *Shewanella decolorationis* S12 occurred after 36 hours, with electron donors [18]. Recently, the decolourisation of Amaranth under microaerophilic condition by a bacterial consortium NAR-2 within 30 minutes at 45°C has also been reported [15]. These suggest that decolourisation rate of Amarath L17 is comparable to the other azo dye decolourising bacteria reported.

3.2 Decolourisation of Amaranth under Microaerophilic Condition in Minimal Media

The minimal media used in this study have lower amount of glucose, except MMP5, which is devoid of both glucose and nutrient broth. In all minimal media, full decolourisation of Amaranth was observed at 37°C. Decolourisation was found to be the fastest in MMGF11, and the slowest in MMP5 (Figure 2a). Experiment performed at 45°C showed that L17 requires longer time to fully decolourise the azo dye in the three minimal media (Figure 2b), though the fastest decolourisation observed was still in MMGF11. On the other hand, only partial decolourisation could be observed in MMP5. It was found that 37°C is the most suitable temperature for microaerophilic decolourisation by L17 in all media.

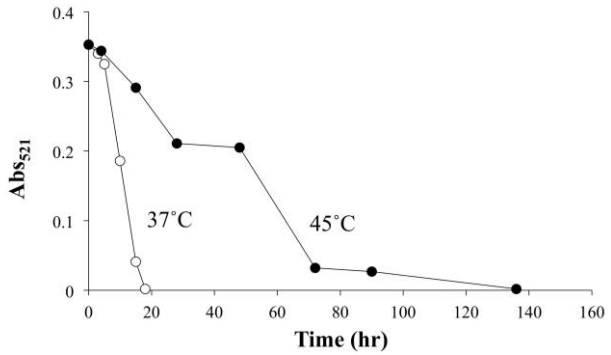


Figure 1 Decolourisation of Amaranth by L17 at 37°C and 45°C under microaerophilic condition in MP5 medium

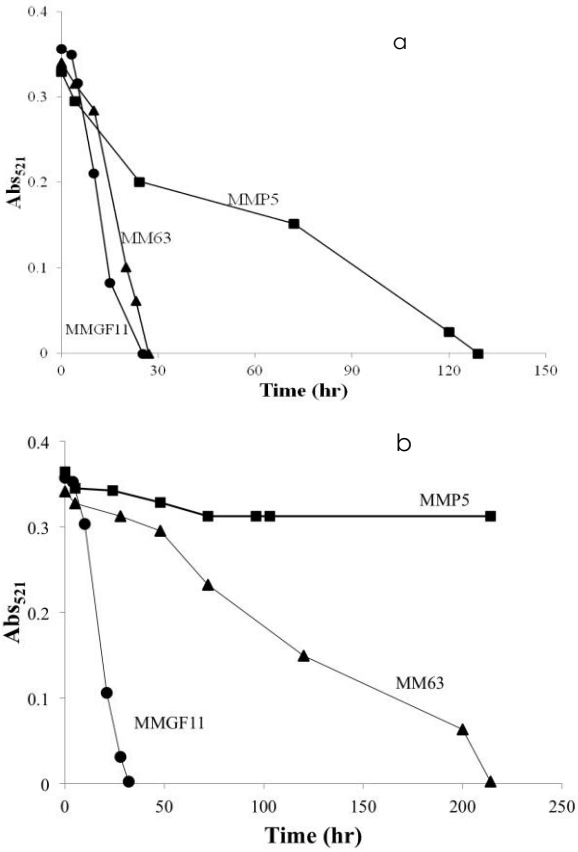


Figure 2 a) Decolourisation of Amaranth by L17 in minimal media at 37°C and b) at 45°C under microaerophilic condition

3.3 Decolourisation of Amaranth under Anaerobic Condition in Rich Medium

Anaerobic condition was achieved by purging the medium with nitrogen gas. The serum bottles were incubated at 37°C after inoculation of L17 and the decolourisation was observed. The reaction temperature was chosen based on the results obtained from experiments performed under microaerophilic condition. Amaranth in MP5 (rich medium) was decolourised fully after 17 hours (Figure 3). Generally, microbial degradation of azo dyes

involves the reductive cleavage of azo bonds with the help of an azoreductase enzyme under anaerobic conditions, involving electron transfers. The transfer of electrons to the azo dye, as a final electron acceptor, results in dye decolourisation. The resulting aromatic amines are then further degraded (19, 20].

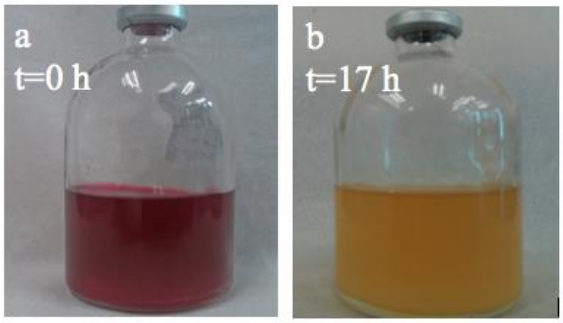


Figure 3 Decolourisation of Amaranth under anaerobic condition by L17 at time zero (a) and after 17 hours (b) in MP5 medium

3.4 Decolourisation of Amaranth under Anaerobic Condition in Minimal Media

In all minimal media used, full decolourisation was observed under anaerobic condition, despite varying duration. The compositions of the media was adapted from other publications, and modified accordingly to investigate the effect of additives such as glucose, vitamin and trace elements onto the extent of decolourisation of Amaranth. Full decolourisation of Amaranth in the minimal media was observed after 24 hours, 26 hours, and 98 hours in MMGF11, MM63, and MMP5 respectively (Figure 4). When compared to microaerophilic condition, decolourisation of Amaranth occurred at a higher rate under anaerobic condition. In the minimal media, the fastest decolourisation occurred in MMGF11, followed by MM63 and MMP5. MMP5 does not contain glucose and/or nutrient broth suggesting that *Citrobacter* sp. L17 has the ability to decolourise azo dye under anaerobic condition, and the presence of glucose at low concentration is sufficient to expedite the process. The presence of vitamin and trace elements does not seem to enhance the decolourisation significantly.

Researchers previously believed that cytoplasmic enzymes were involved in degrading the sulfonated and unsulfonated dyes. But later it was found that it is unlikely for highly charged sulfonated and polymeric dyes to pass through the cell membrane and bind to the enzyme to be degraded.

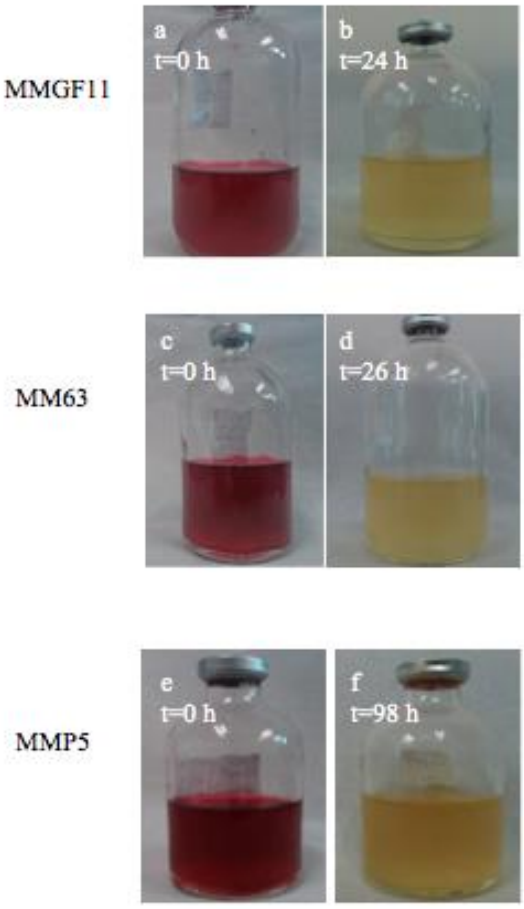


Figure 4 Decolourisation of Amaranth under anaerobic condition by *Citrobacter* sp. L17 at (a) time zero and (b) after 24 hours in MMGF11 medium; (c) time zero and (d) after 26 hours in MM63; (e) time zero and (f) after 98 hours in MMP5

Since then, it has been hypothesized that they are degraded in extracellular space [21]. A wide range of bacteria is able to decolourise azo dyes under strict anaerobic condition [22]. *Citrobacter* sp. L17 has been shown in this study to be able to decoluorise Amaranth under both microaerophilic and anaerobic condition, in rich and minimal media. Knowledge of the decolourisation ability in minimal media under anaerobic condition is critical for future study on biogas production during decolourisation process. An anaerobic condition is generally required for such experiments, and the reaction vessel needs to be gas-tight so that the released gases can be collected and analysed [14].

In the future, the anaerobic decolourisation process will be monitored closely via sampling at regular time interval to investigate the progress of Amaranth decolourisation by *Citrobacter* sp. L17.

4.0 CONCLUSION

In this study, a locally isolated bacterium of gastrointestinal origin, *Citrobacter* sp. strain L17, was explored for its potential to decolourise azo dye under limited oxygen. It was found that the strain has the ability to decolourise Amaranth under anaerobic condition after around 24 hours, in minimal media. Results from this study have suggested a suitable and economical minimal medium (MMGF11) for use in biogas production studies using azo dye as substrate.

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