MCM-41 AS SUPPORT FOR IMMOBILIZATION OF NAR-1 BACTERIAL CONSORTIUM IN THE BIODEGRADATION OF REMAZOL BLACK B

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"Specially dedicated to my father who had passed on. This is a special gift to my mother for her endless love and care".

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ABSTRACT

A novel bacterial consortium, NAR-1 consisting of *Enterococcus* sp. C1 and *Citrobacter* sp. L17 was immobilised onto an inorganic support material and investigated for its biodegradation ability of Remazol Black B (RBB), micro-aerophilically under both batch and fed-batch continuous systems. To study its efficiency under immobilised condition, the NAR-1 bacterial consortium was adsorbed onto a silica mesoporous material, MCM-41 before conducting decolourisation experiments. Successful synthesis of MCM-41 was verified by FTIR, XRD and FESEM. The MCM-41 powder generated was granulated into spheres of 2-4mm in diameter before immobilisation. Preliminary immobilisation of NAR-1 was done conventionally by observing three parameters namely: agitation speed, contact time and operating temperature in 0.85% (w/v) saline. The highest adsorption of bacteria onto MCM-41 granules was 7.8 x 10^5cfu/ml at 100 rpm, 2 hours contact time and37^{o}C.This pre-optimised condition was transferred to RSM for a more precise prediction. RSM predicted a 7.8 x 10^5cfu/ml using an optimised condition of 1.9 h contact time at 34^{o}C and 116 rpm but actual lab experimentusing the above parameters successfully produced a higher immobilised cell count of 9.0 x 10^5 cfu/ml. To compare the reusability of free and immobilised cells with both cell counts fixed at 9 x 10^5 cfu/ml, repeated-batch operation was conducted with constant addition of 100 ppm RBB into Modified P5 medium pH7.0, following each decolourisation cycle. Free cells initially took 240 min in the first cycle, declined to 180 min but yet again elevated to 270 min in the third cycle. It took a staggering 420 min to complete the 4th cycle of decolourisation. Improving decolourisation trend was observed from 180 min to 120, down to half the time at 60 min with immobilised cells. Eleven cycles were completed for immobilised cells as compared to 4 for free cells within a time-frame of 19 h. For continuous fed-batch system in an upflow packed bed reactor, the decolourisation rate progressively escalated from 50% within the first 3 hours to 80 % by the 8th hour. Interestingly, beyond that, stabilised decolourisation at almost 90 % was observed, spanning 28 h covering 9.49 cycles. A prominent decline was noticed after the 36th hour and the efficiency plummeted to 0% by the 56th hour. This sharp decline was conceivably due to several factors including bacterial leach out, bacterial cell death due to toxic accumulation and detachment of biofilm. However, MCM-41, an inorganic material remains as a potential support for bacterial immobilisation and can be applied repeatedly in a continuous system due to its rigidity.

ABSTRAK

Satu konsortium bakteria yang novel, NAR-1 terdiri daripada *Enterococcus* sp. C1 dan *Citrobacter* sp. L17 dijerap pada bahan tak-organik dikaji dalam biodegradasi pewarna azo Remazol Black B secara mikroaerofilik dalam keadaan kultur sesekelompok dan suapan-balik. Keberkesanan keadaan tersekat-gerak dikaji dengan NAR-1 dijerap pada permukaan bahan silika mesoporos MCM-41 sebelum penyahwarnaan. MCM-41 disintesis dan disahkan sifatnya melalui FTIR, XRD dan FESEM. MCM-41 serbuk digranulkan menjadi sfera sebesar 2-4 mm diameter sebelum penjerapan. Eksperimen awal tersekat-gerak secara konvensional dengan NAR-1 melibatkan beberapa parameter seperti kadar agitasi, masa sentuhan dan suhu dalam saline 0.85% (b/i). Penjerapan sel tertinggi direkod pada kadar 7.8 x 10^5 cfu/ml dengan agitasi 100 rpm, masa sentuhan 2 jam dan suhu 37^{o}C. Parameter optimum secara konvensional telah dimasukkan ke dalam program RSM. RSM meramalkan penjerapan bakteria sebanyak 7.8 x 10^5 cfu/ml dengan mengaplikasi parameter optimum iaitu 1.9 jam masa sentuhan, suhu 34 ^{o}C dan kadar agitasi 116 rpm tetapi dengan eksperimen makmal sebenar, ia memberi peningkatan bilangan sel yang disekat-gerak sebanyak 9.0 x 10^5 cfu/ml. Perbandingan penggunaan semula bakteria bebas dan yang tersekat-gerak dengan bilangan bakteria yang ditetapkan pada 9 x 10^5 cfu/ml, eksperimen sesekelompok telah dijalankan dengan penambahan pewarna RBB dengan agitasi 100 ppm ke dalam medium P5 terubahsuai, pH 7.0. Ujikaji dengan bakteria bebas mengambil masa 240 minit pada kitaran pertama, menurun kepada 180 minit dan menaik kepada 270 minit pada kitaran ketiga. Pada kitaran keempat, ia mengambil 420 minit untuk menyahwarnakan RBB. Seterusnya, bakteria yang terjerap di atas permukaan MCM-41 mengambil masa 180 minit pada kitaran pertama dan berkurang ke 120 minit turun kepada 60 minit. Sebelas kitaran penyahwarnaan RBB dicapai dalam masa 19 jam dengan bakteria yang tersekat-gerak berbanding dengan 4 kitaran untuk bakteria bebas. Untuk eksperimen kultur suapan-balik berterusan yang dijalankan dalam reaktor dasar padat aliran menaik, kadar penyahwarnaan RBB meningkat dari 50 % dalam masa 3 jam ke 80 % pada jam yang ke-8. Kadar penyahwarnaan menjadi stabil pada kadar 90 % dalam 28 jam bersamaan 9.49 kitaran. Penurunan yang mendadak pada kadar penyahwarnaan RBB direkodkan selepas jam ke-36 dan seterusnya menjadi sifar pada jam ke-56. Penurunan yang mendadak disebabkan oleh beberapa faktor seperti larut lesap bakteria dari granul, penuaan sel berikutan kandungan toksik meningkat dalam medium dan penanggalan biofilem. Walaubagaimanapun, MCM-41 merupakan bahan inorganik yang mempunyai potensi dalam penjerapan bakteria dan boleh diguna dalam sistem selanjar berulangkali kerana ketahanan yang tinggi.

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LIST OF SYMBOLS / ABBREVIATIONS

CCD	-	Central composite design
g	-	Gram
h	-	Hour
L	-	Liter
M	-	Molar
mg	-	Milligram
min	-	Minutes
mL	-	Milliliter
nm	-	Nanometer
RSM	-	Response Surface Methodology
rpm	-	Round per minute
t	-	Time
T	-	Temperature
v/v	-	Volume per volume
w/v	-	Weight per volume
µm	-	Micrometer
ºC	-	Degree Celsius
%	-	Percentage
NA	-	Nutrient agar
OD	-	Optical Density
A_{600nm}	-	Absorbance at the wavelength of 600 nm
UV	-	Ultraviolet
FESEM	-	Field Emission Scanning Electron Microscopy
XRD	-	X-Ray Diffraction
FTIR	-	Fourier Transform Infrared

CHAPTER 1

INTRODUCTION

1.1 Introduction

Synthetic dyes are extensively used in many industries such as, in various branches of the textile industry, the leather tanning industry in paper production, food technology, agricultural, light-harvesting arrays, photo electrochemical cells, and hair colourings (Van Der Zee and Villaverde, 2004, Ganesh *et. al*., 1994). Unfortunately, the exact amount of dyes produced in the world is not known. It is estimated to be over 10,000 tons per year. Exact data on the quantity of dyes discharged in the environment are also not available. It is believed that a loss of 1–2% in production and 1–10% loss in use are a fair estimate. For reactive dyes, this figure can be about 4%. Due to large-scale production and extensive application, synthetic dyes can cause considerable environmental pollution and are serious health-risk factors. Among the dyes used, azo dyes are the most important and widely used **(**Alexander *et al*., 2002). Azo dyes are characterised by the presence of one or more azo groups (-N=N-), known as the chromophore which gives the dye its colour and negative sulfonate groups ($-SO_3^-$) (Anjali *et al*., 2006). Azo dyes itself is hazardous; however, it can be more hazardous when the azo bonds are reduced to give amines which can be more carcinogenic than the parent structure (Anjali *et al*., 2006).

Currently, numerous methods can be applied for the wastewater treatment of colour removal either through chemical, physical or biological processes. The chemical and physical colour removal processes utilises a number of hazardous

chemical and the by-products are considered non-environmentally friendly. Biological techniques use microbes as powerhouse to directly utilise the azo dyes with no or minimal impact on the environment. Biological techniques for treating dyes employed the use of microorganisms to decolourise and biodegrade azo dyes under anaerobic, aerobic or combined anaerobic/aerobic treatment system. Microbial degradation and decolourisation of dyes is an environment friendly and cost competitive alternative to chemical decomposition processes (Swamy *et al*., 1999, Libra *et al*., 2004 and Rodriguez *et al*., 2002)

Many microorganisms belonging to different taxonomic groups of bacteria, fungi, actinomycetes and algae have been reported for their ability to decolourise azo dyes (Khehra *et al.*, 2005 and Chang *et al.*, 2004). Anaerobic and microaerophilic microorganisms have been found to reduce azo bonds non-specifically in anaerobic conditions leading to dye decolourisation and in the process generate toxic aromatic amines which are mutagenic and carcinogenic (Coughlin *et al.,* 1999). Hence, along with colour removal, complete degradation of azo dyes is the only solution for final elimination of xenobiotics from the environment. Apparently, there exists a need to develop novel biological decolourisation processes leading to more effective cleanup of azo dyes (Padmavathy *et al.*, 2003).

In this research, an azo dye Remazol Black B (RBB) or also known as C.I. Reactive Black 5, in particular was decolourised using selected bacterial consortium NAR-1 comprising of *Enterococcus* sp. (C1) and *Citrobacter* sp. (L17). *Citrobacter* sp. L17 was previously reported as *Enterobacter cloacae* L17 (Chan *et al*., 2012). However, genome sequence done on these bacteria reidentified the bacteria as *Citrobacter* sp. L17. Therefore, the bacterial strain *Enterobacter cloacae* L17 had been redesignated as *Citrobacter* sp. L17 in this thesis. Previous studies in this lab have shown this bacterial consortium has the ability to reduce RBB anaerobically in 45 mins under batch condition (Ilan Sollan, 2008)**.** The biodegradation products of RBB following reduction process could include aromatic amines and other intermediates such as *p*-base, sulphanilic acid and triaminohydroxynaphthalene disulfonilic acid (TAHNDS). HPLC was used to determine the presence of

sulphanilic acid as it was previously reported to be a dead end product in RBB biodegradation (Alexander *et al.,* 2002).

A type of mesoporous silica material called MCM-41 (Mobil Carrier Material; Mobil Catalytic Material) was utilised as a bacterial support for the degradation of RBB in this research. Mesoporous silica is a form of silica and a recent development in nanotechnology. The most common type of mesoporous nanoparticle is MCM-41 (Amit *et al.,* 2006). Research continues on MCM-41 which has applications in catalysis, drug delivery and imaging (Brian *et al.,* 2007). A procedure for producing mesoporous silica was patented around 1970 (Chiola *et al.,* 1971). It went almost unnoticed and was reproduced in 1997 (Zu *et al.,* 2007 and Direnzo *et al.,* 1997). Mesoporous silica nanoparticles (MSNs) were independently synthesised in 1990 by researchers in Japan (Tsuneo*et al.,* 1990). They were later produced at Mobil Corporation laboratories and named Mobil Crystalline Materials, or MCM-41 (Beck *et al*, 1992 and Trewyn, *et al.,* 2007). The researchers who invented these types of particles planned to use them as molecular sieves. Today, mesoporous silica nanoparticles have many applications in biotechnology, medicine, biosensors, and imaging. In this research, a novel approach was taken whereby, MCM-41 was utilised as bacterial support in the bioremediation of azo dyes.

Response Surface Methodology (RSM)is important in designing, formulating, developing, and analyzing new scientific studying and products. It is also efficient in the improvement of existing studies and products. The most common applications of RSM are in industrial, biological and clinical science, social science, food science, and physical and engineering sciences (Nuran, 2007). Since the conventional method of optimisation, ‘‘one factor at a time’’ approach is laborious, time consuming and incomplete, RSM using CCD (as factorial experimental design) which involves full factorial search by examining simultaneous, systematic and efficient variation of important components was applied to model the cell adsorption process on MCM-41 granules. The method identifies possible interactions, higher orders effects and determines the optimum operational conditions. However, RSM using CCD is useful for small number of variables (up to five) but is impractical for a large number of

variables, due to high number of experimental runs required (Sharma and Satyanarayan, 2006).

Packed bed reactors are well-known to be utilised in various applications of biotechnology. Particularly, attached biofilm reactors present a higher potential for use than suspended growth biomass reactors because the former can retain higher concentrations of biomass with higher metabolic activity. Moreover, attached biomass is known to be more resistant to toxicity. So, the ultimate objective of this study was to evaluate the performance of a biofilm packed bed reactor for the removal of dye.

1.2 Problem Statement

Batch decolourisation of RBB by NAR-1 done in this lab had been successful but only small reaction volumes could be handled at one time. Therefore, a continuous decolourisation system requiring a support material for bacterial adhesion is necessary. A robust yet inert support without the need to be modified could simplify the process. Hence, MCM-41 was the choice of support material as it has met the above requirements. This was granulated, loaded into an upflow packed bed reactor and used in the immobilisation of NAR-1, followed by continuous RBB decolourisation process.

1.3 Objectives of Study

The objectives of this study were:

a. To synthesise and characterise ordered mesoporous silica (MCM-41) and granulate MCM-41 powder.
b. To optimise bacterial adsorption process on MCM-41 granules using conventional method and subsequently RSM.

c. To compare repetitive batch decolourisation of RBB using suspended bacteria or bacteria immobilised onto MCM-41.
d. To decolourise RBB continuously by NAR-1 immobilised onto MCM-41 granules in a packed-bed reactor.

1.4 Scope of Study

The scope of this research was to utilise ordered mesoporous silica (MCM-41) which was synthesized in this lab as support for immobilization of a bacterial consortium, NAR-1 for RBB decolourisation. The bacterial strains used were *Enterococcus* sp. C1 and *Citrobacter* sp. strain L17. The research also employed both conventional and RSM approaches to optimize batch immobilisation of NAR-1 onto MCM-41 granules. Additionally, the project concentrated on the feasibility of the packed-bed reactor for the attachment of bacterial cells and consequently continuous decolourisation of RBB.

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