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Research Article

CLONING AND EXPRESSION A RECOMBINANT AMIDASE FROM *RHODOCOCCUS* UKMP-5M

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ABSTRACT
Amidase is a useful enzyme in industries for the synthesis of carboxylic acids, hydroxamic acids and hydrazide which applied in pharmaceuticals, agrochemicals, waste water treatment and commodity chemicals production. In this study, the amidase gene from Malaysia rhodococcal strain, *Rhodococcus* UKMP-5M was cloned by PCR amplification with primers developed by use of genomic sequence of the strain. The amidase gene was expressed in *E.coli* host strain, Rosetta-gami™ (DE3) at 16°C and 20°C for 16 hours with 0.5mM IPTG induction. The expressed amidase represent a protein size of 56 kD on the SDS-PAGE electrophoresis and confirmed by Western blot analysis.

Keywords: *Amidase, Rhodococcus*

INTRODUCTION
The genus *Rhodococcus* is a very diverse group of bacteria that possesses the ability to degrade a large number of organic compounds. Many applications are found in the environmental, pharmaceutical and chemical sectors. Rhodococci are well-established industrial biocatalyst which recently gained an increasing interest in industry due to their mild conditions (physiological pH and ambient temperature), environmentally attractive catalyst, high activities and inherent excellent selectivities (Bell *et al.,* 2009).
Amidases involved in the metabolism of nitriles and amides in bacteria belong to a group of acylamide-amidohydrolases (EC 3.5.1.4) catalyzing hydrolysis of amides to free carboxylic acids and ammonia. It is involved in nitrogen metabolism in cells and widely distributed in nature, commonly found in both prokaryotic and eukaryotic organism. The microbial degradation of nitriles proceeds via two different enzymatic pathways. Firstly, nitrilase, cyanide hydratase and cyanide dihydratase catalyze the direct hydrolysis of nitriles into the corresponding carboxylic acid and ammonia and secondly, nitrile hydratase convert nitriles to the corresponding amide which is then hydrolyzed to the corresponding carboxylic acid and ammonia by an amidase (Yamada and Kobayashi, 1996). These enzymes have been recognized as catalyst of choice in various novel biocatalytic processes to find alternatives to the conventional acid- or base-catalyzed nitrile hydrolysis, a process that due to harsh conditions which is often incompatible with the sensitive structures of many industrially relevant compounds (Martinkova *et al.,* 2009).
Amidase have a great potential for the production of a range of industrially useful amide and acid products like carboxylic acids, hydroxamic acids and hydrazide which find applications in commodity chemicals synthesis like acrylamide, pharmaceuticals, agrochemicals and waste water treatment (Banerjee *et al.,* 2002). Thus, the aim of this study is to express the rhodococcal amidase gene in the heterologous expression system that will contribute in the production of biocatalyst, enantiopure amides and carboxylic acids.

MATERIALS AND METHODS
Bacterial Strain and Plasmid
Strain *Rhodococcus* UKMP-5M was locally isolated from a petroleum contaminated soil and maintained at the Universiti Selangor Culture Collection Centre, Shah Alam, Malaysia. This bacterium was grown in as a source of the amidase gene. Vector pGEMT (Promega) was involved in cloning PCR fragments and

vector pET32b (Novagen) served as an expression vector. *E.coli* JM109 served as a host for cloning and *E.coli* Rosetta-gami™ (DE3) (Invitrogen) as an expression host.

Plasmid Construction and Transformation

The amidase gene was amplified according to the nucleotide sequence of amidase gene from *Rhodococcus* UKMP-5M genome contig where an open reading frame of 1038 bp, encoded a putative polypeptide of 345 amino acid residues was identified. Nucleotide sequence analysis of this gene revealed high homology with aliphatic amidases from rhodococcal bacteria. Sequence comparison of this gene to the amidase gene from several *Rhodococcus* in the genbank resulted in 90% similarities at DNA level and 98% identity at the amino acid level. The amidase gene was amplified using the PCR primers Amd_UKM F 5' ggatccatgcgacacggagacatctcctcc a 3' and Amd_UKM R 5' aagcttctacgcctcggcggtctcttc 3'. The PCR product was cloned into a cloning vector, pGEMT using the pGEMT cloning kit (Promega) and was designated as pAmd. To construct the amidase gene for the expression, the amidase gene from pAmd was subcloned into an expression vector pET32b (Novagen) in which the expression of amidase gene is under control of T7 lac promoter.In pET32b, the gene was fused upstream to a Trx tag for protein solubility and downstream with His tag for protein purification (Figure 1). The constructed gene finally transformed into *E.coli* host strain, Rosetta-gami™ (DE3) designed as pAmd_RG for gene expression.

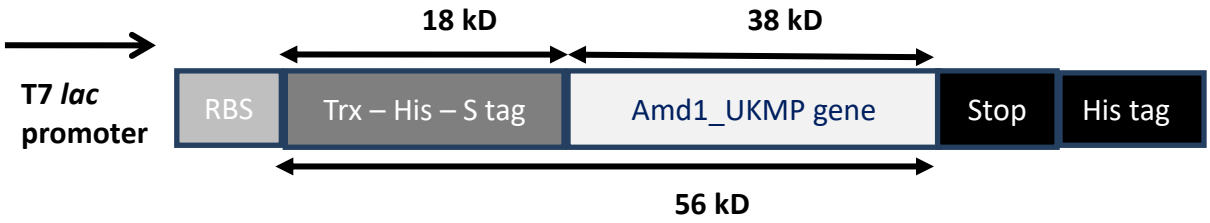

Figure 1: The Recombinant Plasmid pAmd with trx-His-S Fusion Tag and T7 Lac Promoter for Gene Expression and Purification

Amidase Expression

E. coli Rosetta-gami™ (DE3)/pAmd_RG was grown overnight at 30°C in LB broth supplemented with 100µg/mL ampicillin. The overnight culture was centrifuged (10min, 1300g), washed in sterile Ringers solution, and adjusted to an OD_{600} of 1.0. Ten milliliters of the washed overnight culture was used to inoculate 500 mL LB broth containing 100µg/mL ampicillin which was incubated at 37°C with shaking (200rpm) until the OD_{600} reached 1.0. The cells were cooled to 20°C and IPTG was added to a final concentration of 0.5 mM and the culture was incubated for overnight at 20°C with shaking (200rpm). The induced culture was centrifuged at 5500g for 20 min at 4°C. The cell pellets was resuspended in 10 mL ice-cold lysis buffer (50mM sodium phosphate pH 7.4 1 mM phenylmethylsulfonyl fluoride (PMSF), 1mM EDTA and 5% glycerol). The cells were lysed using five sonication-cooling cycles (on ice) in a MSE Soniprep 150 with a microtip. The sonicated cellswere centrifuged at 10,000g for 30 min at 4°C. Crude protein from lysate and soluble supernatant from the cell pellets was analysed by SDS-PAGE gel electrophoresis and confirmed by Western blotting.

RESULTS AND DISCUSSION

A fragment of the amidase gene was cloned by PCR amplification using primers developed on the basis of genome contigs sequence of *Rhodococcus* UKMP-5M. The 1038 bp ORF of the gene was ligated into pET32b expression vector by sticky end cloning upstream to a Trx tag and downstream with His tag and was designed as pAmd plasmid. A database search of the amidase gene sequence revealed high homology of sequences of a number of aliphatic amidases. The plasmid was designed so as making the expression protein soluble by the thioredoxin (Trx tag) which enhance protein solubility and allow the formation of disulfide bonds which are required for proper folding and protein activity while the His-tag is for protein purification by affinity chromatography.

The recombinant plasmid, pAmd was transformed into *E.coli* host strain, Rosetta-gami™ (DE3) where the expression was tightly regulated by the T7 *lac* promoter and corrected the rare codon usage by the host strain. TheSDS-PAGE of crude extracts of supernatant and soluble supernatant of the recombinant protein was approximately 56 kD in size. It was produced at temperature 20°C and 16°C for overnight or approximately 16 hoursin the absence of self promoter and after induction with IPTG (Figure 2). However, before using *E. coli* Rosetta-gami™ (DE3) host strain, the amidase gene was not expressed in ordinary expression host *E. coli* DE3 due to several rare codons usage in the gene that failed the expression. Thus, to overcome the problem we used *E. coli* Rosetta-gami™ (DE3) host strain which could corrected the rare codon for heterologous expression.

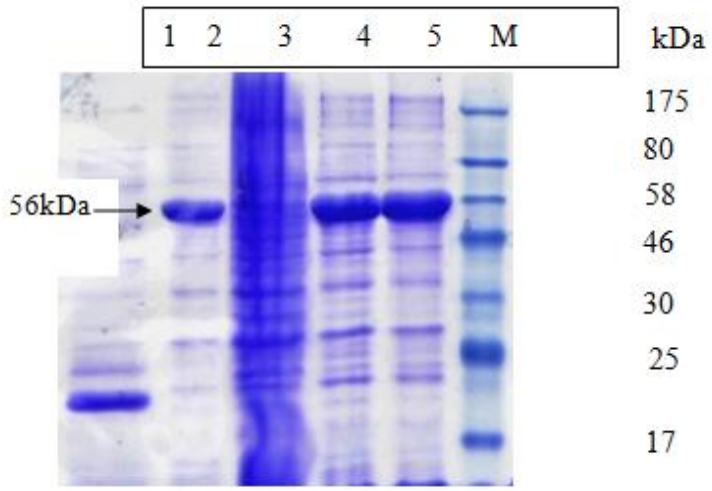

Figure 2: SDS-PAGE of Crude Extracts of Cultures Grown and Induced with 0.5 mM IPTG at a Variety of Temperatures and Incubation Time; Lane M: NEB Protein Marker; Lane 1: Uninduced pAmd_RG as a Negative Control; Lane 2: pAmd_RG;30°C/4hrs; Lane 3: pAmd_RG;25°C/16hrs; Lane 4: pAmd_RG; 20°C/16hrs; Lane 5: pAmd_RG;16°C/16hrs

The recombinant protein was extracted by sonication and centrifuged at 10 000rpm for 20 minutes at 4°C and clear lysate was subsequently purified using HisTrap AKTAPRIME purification for enzyme characterization. Kinetic analysis of the enzyme has been carried out to determine substrate specificity and catalytic efficiency towards various amides.

Conclusion

The amidase gene of *Rhodococcus* UKMP-5M has been expressed in *E. coli* Rosetta-gami™ (DE3) for rare codon expression.

ACKNOWLEDGMENT

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