Effect of Molecular Mutagenesis on Alcohol Dehydrogenase Production by *Pseudomonas fluorescens* RB30

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Abstract

Alcohol dehydrogenases are group of dehydrogenase enzymes that occur in many organisms and facilitate the interconversion between alcohols and aldehydes or ketons with the reduction of NAD^+ to NADH. Locally isolated *Pseudomonas fluorescens* RB30 was selected depending on its efficiency in producing of alcohol dehydrogenase (ADH) enzyme. Its specific activity was 13.6 U/mg proteins after extraction by using lysozyme. As an attempt to improve ADH production, *Pseudomonas fluorescens* RB30 was subjected to molecular mutagenesis following conjugation with *E. coli* PULB113, Tet^r and Km^r harboring the conjugative plasmid RP4 that carried Mini-Mu transposon, and two mutants were due to their high activities were resulted (0.82 U/ml and 0.79 U/ml respectively).

Keywords: *Pseudomonas,* alcohol dehydrogenase, molecular mutagenesis

Introduction

Alcohol dehydrogenase (ADH) enzyme is a primary defense factor against alcohol; which is the toxic molecule compromises the function of human nervous system [1]. Alcohol is usually converted to acetaldehyde, an even more toxic molecule, which is quickly further converted into acetate and other molecules that are easily utilized by human cells. Therefore, a potentially dangerous molecule is converted through ADH into more food stuff [2]. ADH is one of the four distinct groups of related enzymes that have the same action which catalyzes oxidation of alcohols to carbonyl compounds by utilizing either NAD^+ or $NADP^+$ as cofactor. These are ADH, L-lactatedehydrogenase, malatedehydrogenase and glyceraldehydes-3-phosphate dehydrogenase [3].

In bacteria, metabolism of n-alkanes is generally thought to follow a pathway in which the terminal methyl groups sequentially oxidized to the corresponding primary alcohol, aldehyde and acid [4]. The first step in this oxidation pathway is an oxygen dependant reaction catalyzed by monooxogenase system (by presence of ADH enzyme) [5, 6]. Researches on bioconversion and degradation of oil and oil fractions have lately received a great deal of attention [7, 8, 9]. Microorganisms are able to use specific oil fractions as a carbon and energy source or converting oil component to useful products [7, 10, 11].

Pseudomonas could be isolated from petroleum contaminated areas [12]. It possesses different enzymatic systems like monoterminal oxidation which ADH is one of important enzyme, and subterminal oxidation system for aliphatic hydrocarbon. Besides the catabolic pathways that cleave and oxidize the aromatic hydrocarbons through *ortho, para* and *meta* pathways [12, 13]. ADH is also playing a control role in the most ancient business of biotechnology; it performs the last step in the conversion of food into metabolic energy creating ethanol instead of detoxifying it [14].

The importance of ADH was clearly identified. However, many attempts were made to increase the yield of ADH especially that by extracted from *Pseudomonas* spp. Molecular mutagenesis by transposable elements (Mini-Mu) is one of these methods. Transposable elements are regulative elements that transpose from one site to another and so infecting gene expression [15].

Mini-Mu transposon is a mutant transposable element derived from Mu phage DNA. The Mini-Mu transposon is carried on

the RP4 conjugated plasmid of *E.coli* PULB113 [16, 17]. These Mini-Mu elements have selectable genes for ampicillin or kanamycin or both [18], Mini-Mu has the ability to jump and randomly inserts in the recipient DNA cell to produce a random mutation that can be induced by heat induction technique [17, 19].

This study was amid to increase the ability of *Pseudomonas fluorescens* RB30 in alcohol dehydrogenase production by molecular mutagenesis using Mini-Mu transposon.

Materials and Methods

Bacterial Isolates: Four *Pseudomonas aeruginosa* isolates were used in this study (*P. aeruginosa* RB19, *P. aeruginosa* RB31, *P. aeruginosa* RB27, *P. aeruginosa* RB29 and *P. fluorescens* RB30). They are wild type obtained from a previous study [22], and *E. coli* PULB113 (Tetracycline resistant and Kanamycin resistant) was obtained from the Department of Biotechnology/Baghdad University.

Enzyme Extraction: Each *Pseudomonas* isolate (RB31, RB19, RB27, RB29 and RB30) were grown in 250ml of salts medium [6] which contain: 5gm $(NH_4)_2SO_4$, 5gm K_2HPO_4, 1gm NaCl and 0.2gm $MgSO_4.7H_2O$ supplemented with 0.1% yeast extract, 0.3% peptone and 1% glycerol. The contents dissolved in 1L distilled water, pH adjusted to 7.2 and sterilized by autoclaving at 121 ^oC for 15min. The cells were incubated at 37 ^oC for 24hrs. The enzyme was extracted by Schwichamer method [23]. After harvesting the cells by centrifugation at 6000 rpm for 20min., the supernatant was discarded and the pellet washed twice with washing buffer [6] then resuspended in the same volume of TE buffer. Lysozyme was added to cell suspension with a final concentration of 0.001gm/ml and the mixture was incubated in shaker water bath at 37^{o}C. For more disruption of cells an osmotic shock was performed by adding 50µl of NaOH solution (0.1N) to the sample [24]. When more than 99% of the cells were disrupted after 20min., immediate transfer the sample to an ice bath. The suspension was centrifuged at 6000 rpm at 4 ^{o}C for 20min and the supernatant was used to determine the ADH activity.

Enzyme Assay [6]: A portion of 3ml of substrate solution (Sodium potassium phosphate 10mM, Dithiotheretol 1mM, NAD^+ or $NADP^+$ 1mM and Ethanol as substrate 3mM) was placed in the cell of a spectrophotometer, and appropriate quantity (50µl) of crude or partial purified enzyme was added to the reaction solution and mixed gently. The ADH activity was routinely monitored as the increase in the absorbance at 340nm during 3min.

Enzymatic activity in U/ml was preformed according to the above method using the following equations:

Activity (U/ml) = $3.02/\epsilon \times 1.0 \times 0.05 \ .\Delta E/min.$

When $\epsilon_{340} = 6.22$ [cm³/µmol].

The Specific Activity U/mg = Volume Activity/Protein Concentration.

Protein concentration was determined according to the method described by Bradford [20].

Bacterial Conjugation: First, by the bacterial conjugation which was performed between the donor strain (*E. coli* PULB113, Tet^r and Km^r harboring the conjugative plasmid RP4 that carried Mini-Mu transposon) and the recipient isolate *P. fluorescens* RB30 (Tm^r, Neo^r and Amp^r) [22] in order to transfer the RP4 to *P. fluorescens* RB30 [25].

Molecular Mutagenesis: The second step involves growing the transconjugant colonies of *P. fluorescens* RB30 that resulted from conjugation experiment in 10ml of LB broth at 37 ^{o}C for 24hrs. After incubation, the culture was subjected to heat induction at 40 ^{o}C for 90min to induce the Mini-Mu transposable element for transposing from RP4 plasmid and insert itself in the chromosome randomly [21]. After that, 0.1ml of each appropriate dilution was spared on LB agar medium and incubated at 37 ^{o}C for 24hrs. After that the colonies were selected and checked to produce ADH enzyme.

Results and Discussion

Detection of ADH from *Pseudomonas* spp.: Five isolates *P .aeruginosa* (RB19, RB31, RB27, and RB29) and *P. fluorescens* RB30 were isolated in previous study [22] from area contaminated with crude oil and its derivatives. The isolates were showed good ability to utilize crude oil and a wide range of hydrocarbon compounds [22]. All *Pseudomonas* isolates were screened to investigate their ability to produce intracellular ADH enzyme. After growing the isolates under same conditions and on the same carbon source (glycerol), various specific activity of ADH were obtained between these isolates (Fig.(1)).

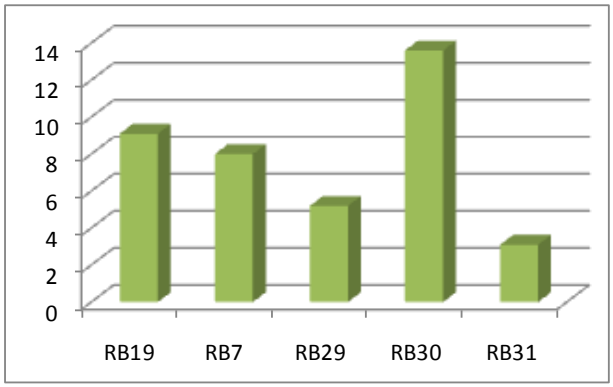

Fig.(1) Ability of Pseudomonas Isolates in Production of ADH. Growing in Salt Medium (pH: 7.2) with 1% Glycerol Substrate at 37^{oC} for 24hrs.

In addition *P. fluorescens* RB30 possessed the highest intracellular ADH specific activity among others, which its specific activity was 13.6 U/mg proteins while the others showed lower specific activity. As a result, the isolate RB30 used for molecular mutagenesis in an attempt to increase the ADH production.

Effect of Molecular Mutagenesis on *P.fluorescens* RB30: Molecular mutagenesis by Mini-Mu transposon of *P. fluorescens* RB30 was carried out in order to obtain more efficient strains to be used for conjugation with *E. coli* PULB113. Many transconjugants of *P. fluorescens* RB30 were isolated on the selective media which based on the genetic markers carried on both conjugative plasmid RP4 (Km^r, Tet^r) and on *P. fluorescens* RB30 (Tm^r, Neo^r and Amp^r).

After one of the selected transconjugants was grown, it was subjected to transposon mutagenesis by heat induction to Mini-Mu transposon at 40 ^oC for 90 min. Culture was spread on the selective medium and ten colonies (mutants) were selected and screened for the ability to produce ADH enzyme. Results (Table (1)) showed that the effect of molecular mutagenesis was varied. Mini-Mu was led to increase the ability of *P. fluorescens* RB30 to produce ADH. Two mutants (RB30-2, RB30-8) were characterized by their high efficiency of ADH production. The specific activities for the enzyme were 19.8 U/mg and 19.7 U/mg proteins, respectively, in comparison to 14.0 U/mg proteins for the wild isolate. Adversely, Mini-Mu also caused decrease in ADH production in the other mutants.

It can be indicated that heat treatment led Mini-Mu to transpose and inserted itself randomly in different sites on the chromosome. Moreover, this caused inactivation of genes that previously subjected to insertion by Mini-Mu. Tossaint and Resibios [16], were found that the Mini-Mu transposable elements which were derived from Mu phage capable to transpose and cause random mutation by one of genetic modification mechanisms (transposition, deletion or duplication of host fragment chromosome) [14, 26].

Insertion of Min-Mu may be occurred in the regulatory gene sites that may regulate the production of ADH negatively, and, hence, this may lead to high level production of the enzyme for the two mutants RB30-2 and RB30-8. While the decrease in enzyme production of other mutants may be attributed to the insertion of Mini-Mu in the structural genes sites coding for the enzyme protein, and then led to decreasing the production of ADH.
From the above finding, heat treatment led Mini-Mu to transpose and insert itself randomly in different sites on the chromosome which inactivated the genes that subjected to insertion by Mini-Mu. The Mini-Mu transposable elements were derived from Mu phage [16] that capable to transpose and cause random mutation by one of genetic modification mechanisms (transposition,

deletion or duplication of host fragment chromosome) [14, 26].

Table (1)
Effect of Molecular Mutagenesis on ADH Production from P. fluorescens RB30.

Mutant No.	*Activity (U/ml)*	*Specific Activity (U/mg Protein)*
Control RB30	0.42	14.0
RB30-1	0.39	13.0
RB30-2	0.82	19.8
RB30-3	0.42	14.0
RB30-4	0.39	13.0
RB30-5	0.39	13.0
RB30-6	0.39	13.0
RB30-7	0.29	10.0
RB30-8	0.97	19.7
RB30-9	o.42	14.0
RB30-10	0.41	13.6

Insertion of Min-Mu may be occurred in the regulatory gene sites that may regulate the production of ADH negatively and hence this may led to high level production of enzyme from the two mutants RB30-2 and RB30-8. While the decrease in enzyme production from other mutants may attributed to the insertion of Mini-Mu in the structural genes sites, that codes for the enzyme protein and hence this led to decreasing the production of ADH from these mutants.

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الخلاصة

Alcohol dehydrogenases هي مجموعة من انزيمات الـ Dehydrogenases و الموجودة في عدد من الكائنات و المسؤولة على تحفيز التحول الخارجي بين الكحولات و الالديهايدات او الكيتونات مع اختزال NAD^+ الى NADH. تم اختيار البكتريا المعزولة محليا نوع Pseudomonas fluorescens RB30 خلال هذه الدراسة و ذلك اعتمادا على كفأتها في انتاج انزيم Alcohol Dehydrogenase. حيث كانت الفعالية الخاصة للانزيم ١٣,٦ وحدة / مليغرام بروتين و ذالك بعد استخدام الانزيم الحال في عملية الاستخلاص. و كمحاولة لتحسين انتاج انزيم ADH عرضت بكتريا Pseudomonas fluorescens RB30 للتطفير الجزيئي و ذالك بعد محاولة اقترانها ببكتريا E. coli PULB113 و الحاوية على البلازميد RP4 و الحامل للعنصر القافز Mini-Mu و تم الحصول على طافرتان و اظهرتا فعالية حجمية عالية ٠,٨٢ و ٠,٧٩ وحدة / مليلتر على التوالي.