

The Optimization of Alcohol Dehydrogenase Production from *Pseudomonas aeruginosa* NW

تحديد الظروف المثلى لإنتاج إنزيم Alcohol Dehydrogenase من بكتريا *Pseudomonas aeruginosa* NW

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Abstract

The locally isolated *Pseudomonas aeruginosa* NW was tested for their ability to produce alcohol dehydrogenase (ADH) enzyme by growing the bacteria on three different aromatic hydrocarbons: naphthalene, xylene and toluene. Following five days incubation the bacterium showed a good ability to utilize xylene, limited ability for toluene utilization and failed to degrade naphthalene. The ADH activity was assayed following each day of incubation on xylene as sole source of carbon and energy and it was found that a maximum ADH volume activity was 2.86 and 4.11 U/ml following the third and fourth day of incubation, respectively. The production of ADH was optimized under different conditions involving: temperature, pH, xylene concentration and incubation period. Results indicated that the optimal conditions for ADH production were by incubating the bacterium in MSM medium containing 1% xylene, pH: 8 for four days and incubation at 37°C.

المستخلص

تم اختبار قابلية البكتريا المعزولة محليا نوع *Pseudomonas aeruginosa* NW على إنتاج إنزيم alcohol dehydrogenase (ADH) من خلال تنمية العزلة على ثلاث أنواع مختلفة من المركبات الهيدروكربونية الأروماتية هي النفثالين ، الزايلين ، التلوين . بعد خمسة أيام من الحضانة أظهرت العزلة قابلية جيدة على استهلاك الزايلين و قابلية محدودة على استهلاك التلوين و فشل في استهلاك النفثالين . تم حساب فعالية إنزيم (ADH) بعد كل يوم حضانة بوجود الزايلين كمصدر وحيد للطاقة ، انثبت النتائج أن أعلى فعالية حجمية لإنزيم ADH كانت 2.86 وحدة/مل بعد مضي ثلاثة أيام من الحضانة و 4.11 وحدة/مل بعد مضي أربعة أيام من الحضانة . حددت الإنتاجية المثلى لإنزيم ADH باستخدام عدة عوامل مختلفة منها : درجة الحرارة ، الأس الهيدروجيني ، تركيز الزايلين و فترة الحضانة و أظهرت النتائج أن الظروف المثلى لإنتاج إنزيم ADH هي بتربية البكتريا في وسط غذائي ذي رقم هايدروجيني 8 يحوي على 1% من الزايلين بعد أربعة أيام من الحضانة عند درجة حرارة 37°C .

Introduction

Petroleum is a complex mixture of aromatic and aliphatic hydrocarbons which considered one of the most common environmental contaminants. Microbial enzymes are useful catalysts for the degradation of hydrocarbons and also for the synthesis of added value products in biocatalytic applications [1]. The zinc metalloenzyme alcohol dehydrogenases have a broad specificity, oxidizing a wide range of aliphatic and aromatic alcohols to their corresponding aldehydes and ketones using NAD^+ or $NADP^+$ as coenzymes [2]. ADH is one of the four distinct groups of related enzymes that have the same action which catalyzes the oxidation of alcohols to carbonyl compounds and utilizing either NAD^+ or $NADP^+$. These are ADH, L-lactatedehydrogenase, malatedehydrogenase and glyceraldehydes-3-phosphate dehydrogenase [3]. For the aromatic hydrocarbons, the

conversion of benzyl alcohols to benzaldehydes is catalyzed by an NAD⁺-linked alcohol dehydrogenase. It seems that dehydrogenation is the major route for this transformation; however, the alcohol is presumably oxidized by the monooxygenase to an unstable gemdiol intermediate which is recognizable as the hydrate of the corresponding benzaldehyde [4].

Pseudomonas spp. are widely spread in most petroleum contaminated areas and it's easy to isolated from such areas, for instance *P. aeruginosa* was isolated from beach sand contaminated with oil in Oman [5], also *Pseudomonas* spp. possess different enzymatic systems like monoterminal oxidation (alcohol dehydrogenase is important enzyme within this system) and sub-terminal oxidation system for aliphatic hydrocarbons, besides the catabolic pathways that cleave and oxidize aromatic hydrocarbons through *ortho*, *meta* and *para* pathways [6].

Vangnai and Arp reported that *P. butanovora* can utilize a variety of organic compounds as growth substrates: C₂ – C₉ *n*-alkanes, the corresponding primary alcohols, carboxylic acids and some polyvalent alcohols. In addition butane-grown *P. butanovora* can degrade several chlorinated aliphatic compounds [7, 8].

Many simple aromatic compounds such as benzene, toluene, xylenes, benzoic acid, phenylacetic acid and phenylpropionic acid are degraded by aerobic soil bacteria such as *Pseudomonas* spp. [9]. Unsubstituted aromatics such as benzene, naphthalene, and biphenyl are converted into the corresponding 1,2- dihydroxycatechols via conversion to the *cis*-1,2-dihydrodiol, followed by oxidation. The dihydroxylation reaction is carried out by a family of three-component dioxygenase enzymes comprising: (1) an NADH-dependent flavin reductase; (2) a ferredoxin electron transfer protein containing two Rieske [2Fe/2S] clusters; and (3) a terminal dioxygenase subunit, containing a mononuclear iron (II) cofactor and two further [2Fe/2S] clusters. The subsequent oxidation of the *cis*-dihydrodiol is carried out by a family of NAD⁺-dependent dehydrogenases [10].

The current study was aimed to investigate the ability of *P. aeruginosa* NW to produce ADH and determine the optimal conditions, from temperature, pH, substrate concentration and period of incubation, for ADH production.

Materials and Methods

Bacterial Strain and Culture Conditions

P. aeruginosa NW used in this study was isolated from oil contaminated soil, and was obtained from Biotechnology Department – Baghdad University. The *P. aeruginosa* NW was maintained at 4°C on LB Agar slant [11].

Mineral Salt Medium MSM [12]

This medium was used for the detection of bacterial utilization of different types of pure aromatic hydrocarbon compounds as a sole source of carbon and energy.

Utilization of Pure Aromatic Hydrocarbons

Aromatic hydrocarbons (naphthalene, xylene and toluene) were added to (MSM) in concentration of 0.2%. The medium as 50ml aliquots in 250ml flasks were inoculated with fresh culture (12 hrs incubation) of bacteria after being washed and resuspended in MSM. Cultures then incubated with shaking (180rpm) at 37°C

for 7 days. The growth was determined by measuring the optical density at 600nm wavelength.

ADH Extraction

The ADH enzyme was extracted using a method described by Schwichamer [13]. After harvesting the cells by centrifugation at 6000 rpm for 20min, the supernatant was discarded and the pellet washed twice with washing buffer (10mM sodium potassium phosphate and 5mM MgSO₄.7H₂O), then resuspended in the same volume of TE buffer [11]. 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°C. For more disruption of cells an osmotic shock was performed by adding 50µl of 0.1 N NaOH solutions to the sample. 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°C for 20min and the supernatant was used to determine the ADH activity.

Enzymatic Assay [3]

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 50µl of crude 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:

$$\text{Activity (U/ml)} = 3.02/\epsilon_{340} \times 1.0 \times 0.05 \cdot \Delta E_{340}/\text{min.}$$

$$\text{When } \epsilon_{340} = 6.22 [\text{cm}^2/\mu\text{mol}].$$

$$\Delta E_{340}/\text{min} = (\text{O.D.2} - \text{O.D.1}) / 3\text{min.}$$

$$\text{and } 1.0 \times 0.05 = \text{sample volume.}$$

Characterizing the optimal conditions for ADH Production

Several factors were studied to determine the optimal conditions for ADH production from *P. aeruginosa* NW, which are:

1. Incubation period. An overnight bacterial culture (O.D. 0.6) was used to inoculate 50ml MSM pH: 7.0, containing 0.2% xylene with shaking (180rpm) at 37°C for 7 days. Aliquot (5ml) from the culture was pipetted following 24, 48, 72, 96, 120, 144 and 168 hours of incubation for ADH activity detection.
2. Substrate concentration: Different concentrations of Xylene (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0%) were added to MSM, pH: 7.0. Media were inoculated with an overnight culture of bacteria (O.D. 0.6) and incubated with shaking (180 rpm) at 37°C for 4 days incubation. Cells were harvested for ADH assay.
3. Effect of temperature: An overnight culture of *P. aeruginosa* NW (O.D. 0.6) were grown in MSM, pH: 7.0 media containing 1% xylene with shaking at different temperature values (30, 35, 37, and 40°C) for 4 days incubation. Cells were harvested for ADH assay.
4. Effect of pH: Different pH values were used (6, 7, 8, and 9) by growing the overnight bacterial culture (O.D. 0.6) in MSM, pH: 7.0 media containing 1% xylene with shaking (180 rpm) at 37°C for 4 days incubation. Cells were harvested for ADH assay.

Results

Aromatic Hydrocarbon Utilization

To investigate the effect of carbon source on ADH production, *P. aeruginosa* NW cells were grown in MSM media containing 0.2% of different aromatic hydrocarbons (xylene, naphthalene and toluene) as a carbon source. Results Table (1) indicate that *Pseudomonas aeruginosa* NW cells were unable to grow and uptake naphthalene as a carbon source and slight growth on toluene, however cells showed a significant growth in MSM medium containing xylene. The optical density and ADH volume activity was tested followed each day incubation, using xylene as a substrate. The maximum optical density recorded was (0.46 and 0.54) and the maximum activity recorded was 2.86 and 4.11 following the day 3 and 4 of incubation, respectively.

On the other hand the optical density did not exceed 0.12 following the fourth day from incubation with an ADH activity 1.15, when using toluene as a sole source of carbon and energy.

Table (1): Alcohol dehydrogenase volume activity after the growth of *P. aeruginosa* NW in MSM medium, pH: 7.0 with different aromatic hydrocarbons (0.2%), at 37°C (180 rpm shaking) for 5 days.

Aromatic Hydrocarbons	1	2	3	4	5
	ADH Volume Activity U/ml				
Naphthalene	-	-	-	-	-
Xylene	-	+	++	++	+
		2.89	2.86	4.11	3.61
Toluene	-	-	-/+	+	+
				1.15	1.02

(-) No growth

(+) Slight Growth O.D. = 0.2– 0.4

(++) Good Growth O.D. = 0.41 – 0.6

Optimum Conditions for ADH Production

Several conditions were used to optimize the production of ADH from *P. aeruginosa* NW. The conditions were:

1) Period of incubation: Different incubation periods were used starting from 24 h and to 144 h. Figure (1). Results showed the relationship between the incubation time of *P. aeruginosa* NW and ADH activity. The exponential growth phase was started following 48 hrs with maximum optical density 0.44 (at 600nm) following 96 h. Soon the growth phase was turned into linear phase and the growth started to decrease.

The ADH activity was relatively increased by increasing the growth rate reaching its maximum (4.9 U/ml) after 96 h.

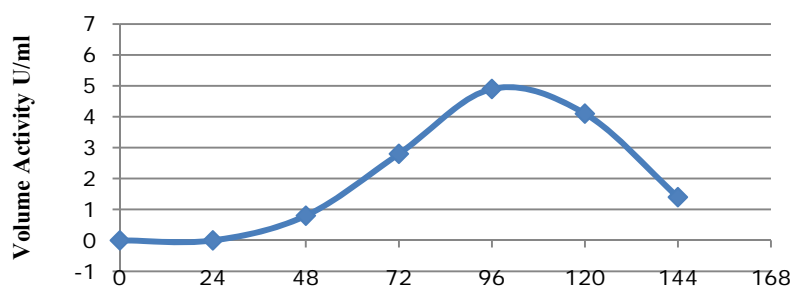


Fig. (1): Alcohol dehydrogenase activity after different incubation period of *P. aeruginosa* with 1% xylene at 37°C (pH=7)

2) Substrate Concentration: Different concentrations of xylene (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2%) were used to determine the optimal concentration. Figure (2), shows that 1% xylene gave the highest ADH activity (8.3 U/ml) after 4 days incubation at 37°C.

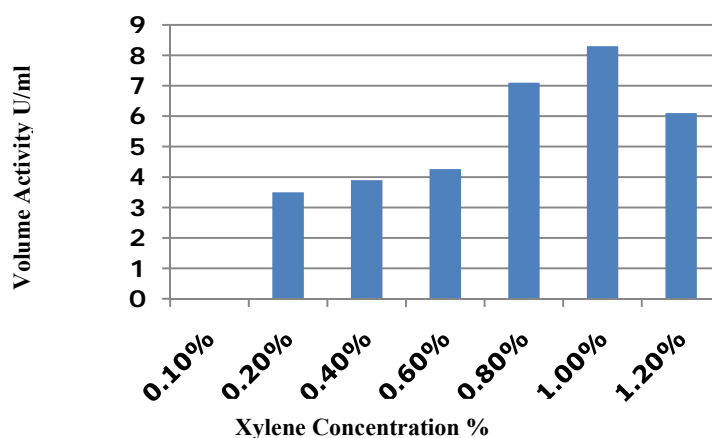


Fig. (2): The effect of different xylene concentration % on ADH production after 4 days incubation at 37°C (pH=7.0).

3) Temperature: The ADH production was examined by incubation of *P. aeruginosa* NW on various temperatures ranges (30, 35, 37 and 40°C). Results Figure (4) revealed that the ADH enzyme showed a maximal activity by incubating *P. aeruginosa* NW on MS media containing 1% xylene at 37°C for 4 days.

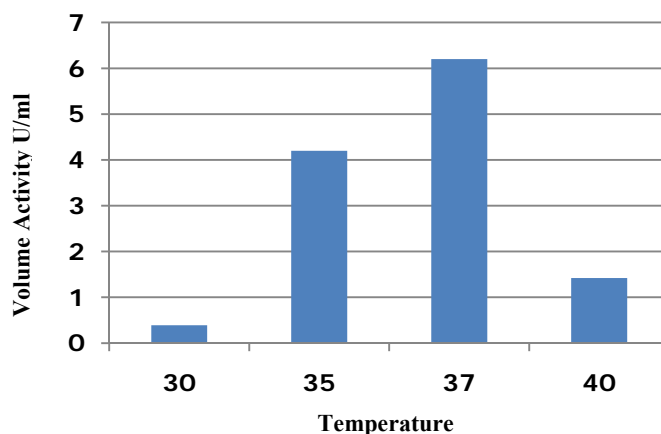


Fig. (3): The effect of different temperature on ADH production after 4 days incubation with 1% xylene (pH= 7.0)

4) pH Value: In order to determine the optimum pH for ADH production, different values of pH were investigated by growing the bacteria *P. aeruginosa* NW in MS medium containing 1% xylene using four different pH values (6, 7, 8, 9). Results in Figure (4) showed that the maximum ADH activity (8.43 U/ml) was observed by growing the bacterium in the media with pH: 8.

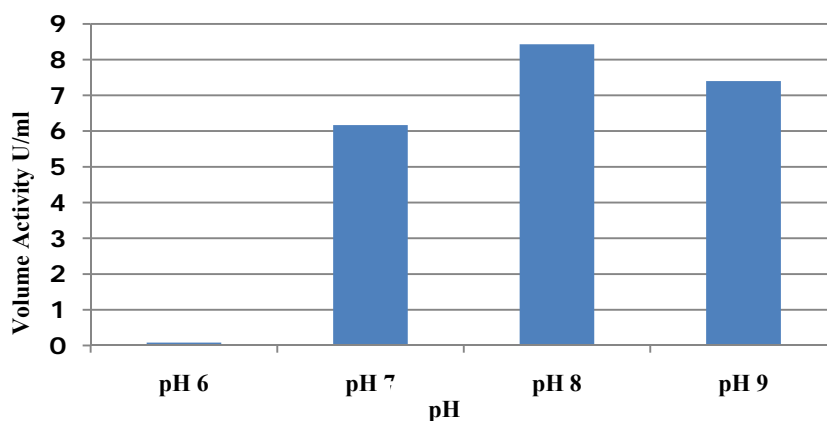


Fig. (4): The effect of different pH values on ADH production after 4 days incubation with 1% xylene at 37°C

Discussion

Results presented above demonstrate that *P. aeruginosa* NW was able to efficiently metabolize xylene and to less extent *P. aeruginosa* NW was showed a slight ability to degrade toluene. Enzymatic assay showed that ADH is one of group of enzymes which involved in aromatic hydrocarbon oxidation and dehydrogenation of benzyl alcohol and benzaldehyde and their *m* and *p*-methyl derivatives are induced only during the growth on the hydrocarbons, the alcohols or the aldehydes [14]. *Pseudomonas* spp. possess different enzymatic systems like monoterminal for aliphatic hydrocarbons [15] besides the catabolic pathways that clave and oxidase aromatic hydrocarbons through *ortho*, *meta* and *para* pathways [16].

The ability of *P. aeruginosa* NW to metabolize xylene and toluene may attributed to the simultaneous conversion of xylene and toluene to toluate via the methyl derivatives, and that further metabolism of these compounds is by the *meta* pathway [16].

The toxicity of toluene against bacteria was previously described [17]. Briefly, toluene is interact with the cytoplasmic membrane leading to the loss of cations Mg^{+2} and Ca^{+2} , as well as other small molecules. In addition to other physiochemical factors (like reduced in solubility and bioavailability) which make toluene not readily biodegradable. This could be explaining the slight ability of *Pseudomonas aeruginosa* NW to uptake toluene.

The reason for not degrading naphthalene may attribute to its complexity and toxicity [18].

Regarding the ADH optimization, results indicated that ADH activity showed its high level after 4 days incubation with 1% xylene. The period of incubation plays an important role in degrading hydrocarbons and it's highly depend on the complexity [19]. According to the Figure (1), the volume activity of ADH showed approximately a semi-logarithmic plot verses time, and reached its maximum following 96hrs of incubation, indicating that a balance in substrate consumption and ADH production took place, in which the cells showed an exponential growth phase which was around 48hrs that allowed the cells to adapt the substrate [20], soon turned into stationary phase with a steady decrease in ADH production and cell turbidity.

Different concentrations of xylene were used to determine which concentration is the best for ADH production. An amount of 1% of xylene was indicated as the best concentration

for ADH production, which reached up to 8.3 U/ml volume activities following 4 days incubation at 37°C. Reports indicated that in order to prevent the toxicity of pure hydrocarbons toward bacterial cells, the hydrocarbon concentration should be maintained between 0.2% [19] to 2% [21], therefore it was considered that 1% is the optimal concentration for ADH production from *P. aeruginosa* NW. It was reported that using 1% of hydrocarbon substrate was gave a high specific activity 19.4 U/mg for ADH production from *Acinetobacter* spp. and *Pseudomonas putida* [3, 22].

The effect of different temperature and pH values were examined on ADH production. As results indicated above, the optimal temperature for ADH production was 37°C and the optimal pH value was 8 following 4 days incubation on 1% xylene. the optimal temperature for ADH production was recorded to be ranged from 35 with optimal value of pH = 7.5 [23], on other hand and as ADH well known for its thermo stability, ADH activity was recorded to be remained up to 50°C, however ADH stability showed a narrow range of pH value ranged between 7.5 – 9 [24].

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