

A New Enzymatic Method for Rapid Diagnosis of Phenylketonuria Using Alkaliphilic *Bacillus*

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Abstract

Rapid and in time diagnosis of phenylketonuria (PKU) in affected infants can help preventing the progress of mental and developmental disorders associated with the disease. Here we report the isolation of alkaliphilic *Bacillus* bacteria capable of producing high level of Phenylalanine dehydrogenase (PheDH) from soil. A new quantitative and rapid test for PKU diagnosis was then developed using the isolated bacteria. The bacterial cells were used to convert serum Phenylalanine to phenyl-pyrovic acid, which was measured quantitatively using spectrophotometer. Application of the method in diagnosis of PKU patients in parallel with the HPLC method produced essentially similar results. However, the cost per sample (10-20 cents per sample) using this new method was much less than that of the HPLC method. The method can be automated which is suitable for mass screening for PKU in populations in which funding is a limiting factor.

Keywords: Phenylketonuria; HPLC; Alkaliphilic; *Bacillus*; Phenylalanine dehydrogenase; Pyrovic acid

Introduction

Phenylketonuria (PKU) is an inborn metabolic disorder with autosomal recessive inheritance. The affected individuals lack the ability to metabolize phenylalanine [1]. If undiagnosed early at birth, patients will develop irreversible brain and developmental disorders. In time diagnosis of the disease and dietary treatment of the patients could prevent the progression of brain damage, and in most cases the normal development can be ensured [2]. One of the traditional diagnostic methods is the Guthrie bacterial inhibition assay (GBIA) [3]. However, this test only distinguishes affected from non-affected individuals and could not

provide a quantitative measurement for serum phenylalanine (Phe). Therefore, in modern countries the test is being replaced by some more rapid, sensitive and quantitative tests, which are commercially available as diagnostic kits [4-7]. These kits are rapid and show very low cross reactivity results and lower false positive, which is associated with the Guthrie test [8]. A number of these commercial kits use L-phenylalanine dehydrogenase (PheDH) (EC 1.4.1.20) enzyme as a reagent in the colorimetric determination of L-phenylalanine in the blood or serum of neonates to detect phenylketonuria [9,10]. Phenylalanine dehydrogenase converts phenylalanine to phenylpyrovate and NH_3 in the presence of NAD^+ , which acts as an electron

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acceptor (Fig. 1) [11,12].

Since the commercial diagnostic kits for PKU are usually expensive, the old GBIA test is still used in most of the laboratories in developing countries, which the money resources are limited. In this study the aim was to find a new simple and affordable quantitative method for PKU diagnosis based on phenylalanine dehydrogenase that can be used easily in our laboratory.

Materials and Methods

Chemical and Reagents

L-phenylalanine was obtained from Sigma Chemical Co., St. Louis, Mo.; Tryptone, Peptone and yeast extracts were purchased from Difco Laboratories, Detroit, Mich. All other chemicals and reagents were from commercial sources and analytical grade.

Media and Culture Conditions

Alkaliphilic *Bacillus* was isolated from alkaline soil (soil sample from a chicken run). The soil sample (0.5 g) was suspended in 5 ml of sterilized water and then heated to 80°C for 15 min. A sample (0.1 ml) of the suspension was spread on Alkaline *Bacillus* medium (containing 1% peptone, 1% glucose, 0.5% yeast extract, 0.1% K₂HPO₄ and 1% Na₂CO₃, the pH was adjusted to 10.8 with 4 M NaOH) and incubated at 30°C on a reciprocal shaker for 2 days. Colonies that can grow on this medium were picked up as Alkaliphilic *Bacillus*. They were maintained individually on solid Alkaline *Bacillus* medium supplemented with 2% agar.

Bacteria and Enzyme Assay

The concentration of bacterial cells in liquid culture media, during logarithmic phase of growth, was estimated by turbidimetry in wavelength equal to 550 nm with a "Zeiss specord S10" spectrophotometer. Enzyme production was estimated by colorimetric method based on measuring the OD of a green-colored ferric complex of phenyl pyrovate at wavelength equal to 614 nm. For this assay, the isolated bacteria were grown on a medium containing 0.1% L-phenylalanine, 0.3% yeast extract, 0.5% NaCl and 0.1% Na₂HPO₄. The pH was adjusted to 10.8 with 4M NaOH. After each bacterial culture reached to 10⁶ of cell number, they were centrifuged at 5000 rpm for 15 min and by adding FeCl₃ to supernatant, the A₅₃₀ of green complex was measured by spectrophotometer. The Ferric complex of phenylpyrovate in the reaction mixture was stabilized using dimethylsulfoxide (DMSO) at 50% v/v [14].

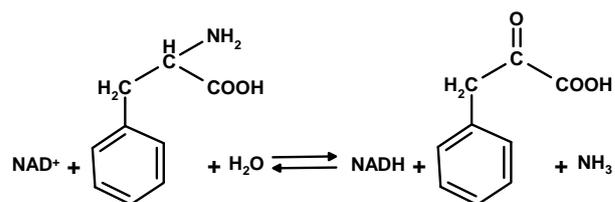


Figure 1. Reaction mechanism of PheDH. Phenylalanine dehydrogenase converts Phenylalanine to phenylpyruvic acid.

Patients and PKU Screening Test

Different concentrations of L-phenylalanine (standards) were prepared as described before [14]. In each test, 500 µL culture media containing 10⁶ bacterial cells was added to 500 µL of each concentration of phenylalanine-BSA solutions. After 5 hours incubation at 30°C, 500 µL DMSO and 200 µL FeCl₃ solution were added. The linearity of the standard curve was analyzed. The concentration of the standards (abscissa) was plotted against the corresponding optical density (ordinate). The standard curve was calculated by a linear regression or a weighted linear regression function.

Blood samples were collected from patients, which were diagnosed positive for PKU in our previous study using Guthrie test [18]. The concentration of phenylalanine in the patient's sera was measured directly from the standard curve upon determination of the optical density of the samples.

Results

Isolation of Alkaliphilic *Bacillus* with High Level of PheDH

In an attempt to isolate alkaliphilic bacteria, 14 strains of these bacteria from alkaline soil that could grow on Alkaliphilic *Bacillus* Medium were identified [15]. The isolated bacteria could tolerate 80°C for 10 min. The isolated bacteria were strict aerobe and spore forming (Cylindrical, central or sub-terminal endospores) gram positive and motile. They were positive for production of catalase and oxidase. The colonies were coded as strain B1 to B14. Only strains B2, B3, B7 and B9 could produce PheDH and could deaminate L-phenylalanine to phenylpyrovate oxidatively [15].

The bacterial cell number in culture media of each strain was counted at different time periods after inoculation by plate count method. When the cell number reached to 10⁶, the optical density of the cultures was determined. This helped to use equal amount of each strain in next experiments by measuring the OD of culture media. The data showed an OD of

0.9, 1.0, 1.0 and 1.2 for 10^6 cells of strains B2, B3, B7 and B9, respectively. This density was found to be virtually constant at the applied condition once the culture was repeated. Therefore, the OD number could be used as an index for determination of cell number in the test experiments.

Analysis of PheDH Activity in Positive Bacterial Strains

The activity of phenylpyrovate produced in 500 μ L of culture media containing 10^6 cells of each strain was determined by measuring the A_{530} of green complex for each strain [14]. The data showed that strains B9 and B3 could produce the highest enzyme activity among the isolated strains (Table 1). The identity of the strains B3 and B9 was examined based on conventional physiological and biochemical characteristics according to Nielsen *et al.* [15,17] (Table 2). Through a series of experiments, the strain B3 was identified as *B. halodurans*, and strain B9 as *B. pseudofirmus* [11,12]. These strains were referred as *B. halodurans* B3 and *B. pseudofirmus* B9 onwards. Generally, alkaliphilic *Bacillus* strains cannot grow below pH 6.5, however, could grow well above pH 9.5. Nevertheless, if sodium ions are supplied at a sufficient high concentration (1-2%) in the medium, the facultative alkaliphilic *B. halodurans* can grow at pH 7 to 10.5. *B. halodurans* is the most thoroughly characterized strain, physiologically, biochemically, and genetically, among those in our collection of alkaliphilic *Bacillus* isolates [11,15].

Analysis of Phe Concentration in PKU Samples

The standard curve for enzyme activity of a 500 μ L of *B. halodurans* B3 culture medium containing 10^6 cells, was drawn. As shown in Figure 2, the standard curve was linear in a range of 0-17.5 mg/dL concentration of Phe ($Y = 0.012x + 0.018$; $R^2 = 0.9989$). The serum Phe from six serum samples from PKU patients was analyzed. Phenylalanine concentration of these samples was previously determined by HPLC method, and therefore could provide a suitable positive control for our method [14]. The test was repeated at least three times for each sample. The data indicated that mean value of each three test were essentially the same as the results obtained by HPLC method as shown in Figure 3.

Discussion

Clinical diagnosis of PKU disease requires a rapid and quantitative test. At present, the most accurate test

Table 1. The level of phenylpyrovate production from isolated strains

| Strain | A_{530} of green complex |
|--------|----------------------------|
| B2 | 0.352 |
| B3 | 0.684 |
| B7 | 0.430 |
| B9 | 0.725 |

Table 2. Taxonomic characteristics of strains B3 & B9

| Characteristic | Strain B3 | Strain B9 |
|---|-----------|-----------|
| Growth at 55°C | + | - |
| Growth in 12% NaCl | + | + |
| Reduction of nitrate | + | - |
| Deamination of phenylalanine utilization of: | | |
| L-Arabinose | + | + |
| D-Xylose | + | - |
| Salicin | + | + |
| Sorbitol | + | - |
| Galactose | + | - |
| Mannose | + | + |
| Rhamnose | + | - |
| Raffinose | + | - |
| Lactose | - | - |
| Gelatin | + | + |
| Starch | + | + |

for PKU diagnosis used in modern laboratories is HPLC [5,6]. However, the HPLC method needs an apparatus that is not affordable in all laboratories throughout the world. Moreover, there are some enzymatic kits available that are expensive especially for Phe mass screening studies. In this study, based on the fact that few Gram-positive and aerobic bacteria such as *Bacillus sphaericus*, *Bacillus badius*, *Sporosarcina ureae*, *Rhodococcus maris* and *Microbacterium* can deaminate phenylalanine to phenylpyrovate in an oxidizing manner, a new method for measuring Phe was developed [11-13]. First, some strains of Alkaliphilic *Bacillus* bacteria, which produce high level of PheDH

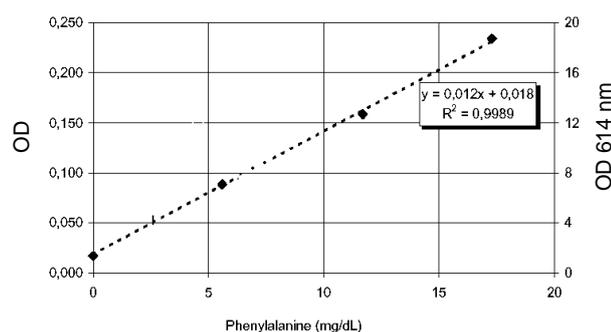


Figure 2. Standard curve for enzyme activity of a 500 µL of *B. halodurans* B3 culture medium containing 10^6 cells.

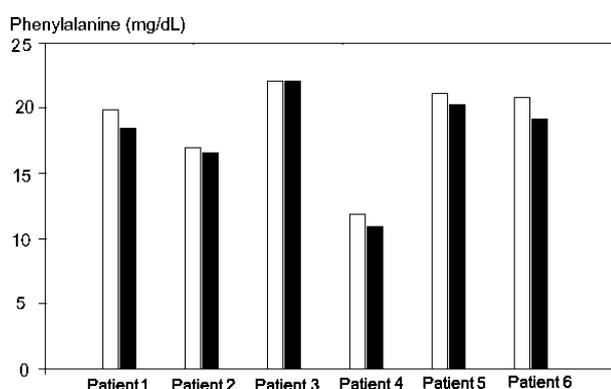


Figure 3. Comparison of results obtained by Alkaliphilic dehydrogenase test with HPLC method results. The white columns indicate the values obtained by HPLC method and the solid columns are the mean values obtained by Alkaliphilic dehydrogenase test.

enzyme, were isolated, and their PheDH enzyme production level was determined chemically. Following isolation of the best strain, a new quantitative test was developed. The test was successfully applied to determine Phe status of serum from 34 PKU patients whose serum Phe was measured using Guthrie bacterial inhibition assay (GBIA) in combination with HPLC [14]. The data showed that the accuracy of the test was comparable with the HPLC [5]. It was noted that usually the values obtained by the new enzymatic test using Alkaliphilic dehydrogenase, were about 0.5 to 2 mg/dL lower than those obtained by HPLC method, which does not interfere with the diagnostic value of the test. Therefore, the new introduced method could be suitable for diagnostic purposes.

The Alkaliphilic dehydrogenase test introduced in this study provides an acceptable accurate test, which has the potential to be developed as a portable kit at its present condition. Moreover, this test provides a

suitable means for mass screening PKU in developing countries in which funding is a limiting factor to perform this kind of screening. Recently, analysis of the entire genome of alkaliphilic *B. halodurans* was completed [16]. This makes this strain suitable for cloning of the PheDH gene. Therefore, it could be possible to produce high level of recombinant PheDH, which can be used in commercial diagnostic kits.

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