International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:6, No:4, 2012

Carbon Sources Utilization Profiles of Thermophilic Phytase Producing Bacteria Isolated from Hot-spring in Malaysia

Noor Muzamil Mohamad, Abdul Manaf Ali and Hamzah Mohd Salleh

***Abstract*—**Phytases (myo-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8) catalyze the hydrolysis of phytic acid (myoinositol hexakisphosphate) to the mono-, di-, tri-, tetra-, and pentaphosphates of myo-inositol and inorganic phosphate. Therrmophilic bacteria isolated from water sampled from hot spring. About 120 isolates of bacteria were successfully isolated form hot spring water sample and tested for extracellular phytase producing. After 5 passages of the screening on the PSM media, 4 isolates were found stable in producing phytase enzyme. The 16s RDNA sequencing for identification of bacteria using molecular technique revealed that all isolates those positive in phytase producing are belong to *Geobacillus spp*. And *Anoxybacillus spp*. *Anoxybacillus rupiensis UniSZA-7* were identified for their carbon source utilization using Phenotype Microarray Plate of Biolog and found they utilize several kind of carbon source provided.

***Keywords*—**Phytase, Phytic Acid, Thermophilic Bacteria, PSM Media and Phytase Assay

I. Introduction

PHYTASES (myo-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8) catalyze the hydrolysis of phytic acid (myoinositol hexakisphosphate) to the mono-, di-, tri-, tetra-, and pentaphosphates of myo-inositol and inorganic phosphate. A broad range of microorganisms, including bacteria, yeasts, and filamentous fungi, produce phytases [1, 2]. Phytic acid is the primary storage form of phosphate in cereal grains, legumes, and oilseeds, such as soy, which are the principal components of animal feeds. However, monogastric animals are unable to metabolize phytic acid and largely excrete it in their manure. Therefore, the presence of phytic acid in animal feeds for chickens and pigs is undesirable, because the phosphate moieties of phytic acid chelate essential minerals and possibly proteins, rendering the nutrients unavailable.

Noor Muzamil Mohamad is with the Faculty of Agricuture and Biotechnology, Universiti Sultan Zainal Abidin, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Malaysia (609 627 5526; fax: 609-627 5633; e-mail: nmuzamil@unisza.edu.my).

Abdul Manaf Ali is with the Faculty of Agricuture and Biotechnology, Universiti Sultan Zainal Abidin, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Malaysia (609-627 5510; fax: 609-627 5633; e-mail: manaf@unisza.edu.my).

Hamzah Mohd Salleh is with the Biotechnology Engineering Department, Kulliyyah (Faculty) of Engineering, International Islamic University Malaysia, Jalan Gombak, 50728 Kuala Lumpur, Malaysia (+603 6196 4495; e-mail: hamzah@iium.edu.my).

Since phosphorus is an essential element for the growth of all organisms, livestock feed must be supplemented with inorganic phosphate [1, 2].

Phytases are of great interest for biotechnological applications, in particular for the reduction of phytate content in feed and food. Enzymes used as feed additives should be effective in releasing phytate phosphate in the digestive tract, stable to resist inactivation by heat from feed processing and storage and also cheap to produce [1, 2]. Thermophilic bacteria are defined as bacteria capable of living at high temperature. Naturally-occuring phytases having the required level of thermostability for application in animal feeding have not been found in nature thus far. Therefore, it comes as no surprise that isolation and characterization and engineering of thermostable enzymes from thermophilic bacteria, as well as the search for the determinants of thermostability are hot spots of current research nowadays [3].

Technology breakthrough in global phenotyping of bacteria had provided better insight on bacteria physiology and metabolism. Recently, Phenotypic MicroArray (PM) profiling and analysis on bacteria have gained popularity. This technology is an integrated system of cellular assays, instrumentation, and bioinformatic software for high throughput screening of cellular phenotype [4]. The knowledge on metabolism of the bacteria and their carbon source utilization profiles will provide valuable data for studying the thermophilic bacteria since the limited research data found on thermophilic bacteria.

The objectives of study were to screen and isolate the thermophilic bacteria that producing the thermostable phytase enzyme. Those positive isolates were recognized using molecular methods. Those isolates will be characterized for different carbon source utilization.

II. Materials and Methods

A. Bacterial Isolation

Water sample were collected from hot spring ponds located in Jeli, Kelantan; Pengkalan Hulu, Perak; Ulu Legong, Kedah; Labis, Johor; Hulu Langat, Selangor and Poring, Sabah. Thermophilic bacteria were isolated and purified using the plate spreading methods and streaking method on nutrient agar (Merck) and incubated at 55°C for 24 to 48 hours. The purification of the single colony of bacteria was done for five passages.

B. Screening of the Bacterial Isolate Producing Extracellular Phytase Enzyme

The isolates obtained from the purification were spread onto modified PSM solid media for screening the phytase producing bacteria as described by S.J. Yoon., et al [5]. This was a synthetic medium consisting of the following (g/L): Calcium phytate 5.0 g; (NH4)2SO4, 3.0 g; MgSO4, 0.5g; CaCl2, 0.1 g; MnSO4, 0.01 g; glucose, 10 g; FeSO4, 0.1 g, made to volume with distilled water and solidified with Bacto agar. The pH was adjusted to 5.5 before autoclaving the media at 121°C, 15 psi for 15 minutes. The inoculate plates were incubated for 3 days at 55°C and the translucent region of the plate gave a visual indication of extracellular phytase production. Any colony thus producing translucent region surrounding the colony indicating extracellular phytase produced will then subcultured to new PSM media. The screening was done for 5 passages to obtain the stable producer for phytate.

C. Characterization and Identification of the Positive Isolates

The isolates those producing phytase enzyme after 5 passages were characterized morphologically, biochemical tests and molecular techniques. The morphology of the isolates were done using Gram staining and endospore staining. The 16s rDNA gene was PCR amplified from genomic DNA isolated from pure bacterial colonies from those positive isolates PCR was done in 25 µL format and containing PCR buffer, 15 mM MgCl2, 0.2mM dNTP mix, 1U of Taq polymerase enzyme, pair forward and reverse universal 16srDNA gene, and genomic DNA from those bacteria as template DNA. The pair of universal primer used in PCR according to Richard Devereux and Sherry S. Wilkinson [6]:

Forward pA: AGAGTTTGATCCTGGCTCAG
Reverse pH: AAGGAGGTGATCCAGCCGCA
Forward 27f: AGAGTTTGATCMTGGCTCAG
Reverse 1525r: AGGAGGTGWTCCARCC

The PCR reaction was run for 35 cycles in a DNA thermal cycler. The following thermal profile was used for the PCR: pre-denaturation at 96 °C for 3 min, denaturation at 96 °C for 1 min, primer annealing at 57 °C for 1 min and extension at 72 °C for 2 min. The final cycle included extension for 7 min at 72 °C to ensure full extension of the products. PCR products were then sent for sequencing service (1st Base, http://www.base-asia.com/). The 16S-rDNA gene sequence of the isolates was aligned with reference 16S-rDNA sequences GenBank using the BLAST algorithm available in NCBI (National Centre for Biotechnology information) in internet.

D. Preparation of PM1 and PM2A

The Isolate number 7 of the positive phytase producing bacteria were inoculated into 12 mL of IF-B Inoculating Fluid to get 80%T (transmittance) using turbidimeter (Biolog, Hayward, USA). IF-B is buffer salt solution which maintains the viability of bacteria cells but does not contain any carbon source. The prepared cell suspension (80%T) was used to inoculate PM1 and PM2A with 100 µL/well. The inoculated PM 1 and PM2A plates then incubated at 55°C for 48 hours and end-point data of the carbon source utilization obtained after 48 hours incubation using GEN III MicroStation System and MicroLog™ 3 Software according to manufacturer instruction.

III. Results and Discussion

About 120 isolates of bacteria successfully isolated from the hot spring water samples. Those isolates were tested using Phytase Screening Media (PSM). This agar media containing inorganic material and glucose and phytic acid (as calcium phytate) as organic material. Any bacteria that produce extracellular phytase enzyme will produce clearing zone due to the breakdown of phytic acid to myo-inositol and phosphate [5].

The bacteria isolates then inoculated onto the PSM media and incubated at 55°C for 3 days. Those positive isolates then again cultured onto PSM media to confirm the stability of production and the survival of bacteria on PSM media. After 5 passages and another 5 passage, only 4 isolates were found stable in producing the enzyme and still survive in PSM media. 4 isolates stable producing phytase design are *UniSZA-3, UniSZA-4, UniSZA-5* and *UniSZA-7*.

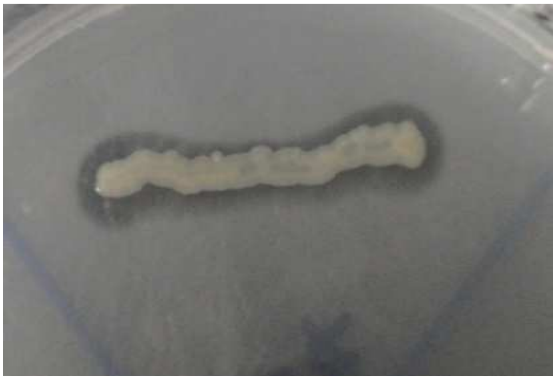

Fig. 1 Screening of phytase positive bacteria by Phytase Screening Medium (PSM) showing halo-zones around the colony

All 4 positive isolates are Gram positive rod with single and chained conformation. All of them producing endospore. The phylogenetic analysis of these strains using its 16S rDNA sequence data showed that Isolates No. 3 had highest homology (99 %) with *Geobacillus sp.*; Isolates No. 4, 5 and 7 showed 99 % similarity with *Anoxybacillus rupiensis*. The isolates of bacteria identified were then design as *Geobacillus sp. UniSZA-3*, *Anoxybacillus rupiensis UniSZA-4*, *Anoxybacillus rupiensis UniSZA-5* and *Anoxybacillus rupiensis UniSZA-7*.

Summary on PM profile of Anoxybacillus rupiensis UniSZA-7 in various substrates, growth temperature (55°C) after 48 hours incubation is shown in Table 1, and 2. Out of 190 carbon source tested (Table 1 and 2), *Anoxybacillus rupiensis UniSZA-7* was found able to utilize a broad spectrum of carbohydrates such as sugars, polysaccharides, polyols, amino acid and other carbon sourced tested.

TABLE I
CARBON SOURCES UTILIZATION PROFILES FOR *ANOXYBACILLUS RUPIENSIS UNISZA-7* ON PM1 PLATE INCUBATED AT 55°C FOR 48 HOURS

Well	Substance	Results
A1	Negative Control	-
A2	L-Arabinose	-
A3	N-Acetyl-D- Glucosamine	+
A4	D-Saccharic Acid	-
A5	Succinic Acid	-
A6	D-Galactose	-
A7	L-Aspartic Acid	-
A8	L-Proline	+
A9	D-Alanine	-
A10	D-Trehalose	+
A11	D-Mannose	+
A12	Dulcitol	-
B1	D-Serine	-
B2	D-Sorbitol	-
B3	Glycerol	+
B4	L-Fucose	+
B5	D-Glucuronic Acid	-
B6	D-Gluc onicAcid	-
B7	D,L-α-Glycerol -Phosphate	-
B8	D-Xy lose	-
B9	L-Lactic Acid	+
B10	Formic Acid	-
B11	D-Mannitol	+
B12	L-Glutamic Acid	-
C1	D-Glucose-6 -Phosphate	+
C2	D-Galactonic Acid -γ -Lactone	-
C3	D,L-Malic Acid	-
C4	D-Ribose	+
C5	Tween 20	-
C6	L-Rhamnose	-
C7	D-Fructose	+
C8	Acetic Acid	+
C9	α-D-Glucose	+
C10	Maltose	+
C11	D-Melibiose	-
C12	Thymidine	-
D1	L-Asparagine	+
D2	D-Aspartic Acid	-
D3	D-Glucosaminic Acid	-
D4	1,2 -Propanediol	-
D5	Tween 40	-
D6	α-Keto –Glutaric Acid	+
D7	α-Keto –Butyric Acid	-
D8	α-Methyl –D-Galactoside	-
D9	α-D-Lactose	-
D10	Lactulose	-
D11	Sucrose	-
D12	Uridine	+
E1	L-Glutamine	+
E2	M-Tartaric Acid	-
E3	D-Glucose -1 - Phosphate	-
E4	D-Fructose -6 - Phosphate	+
E5	Tween 80	-
E6	α-Hy droxy Glutaric Acid- γ- Lactone	-
E7	α-Hydroxy Butyric Acid	-
E8	β-Methyl-D-Glucoside	-
E9	Adonitol	-
E10	Maltotriose	+
E11	2-Deoxy Adenosine	-
E12	Adenosine	-
F1	Glycyl -L - Aspartic Acid	-
F2	Citric Acid	-
F3	M-Inositol	+
F4	D-Threonine	-
F5	Fumaric Acid	-
F6	Bromo Succinic Acid	-
F7	Propionic Acid	-
F8	Mucic Acid	-
F9	Glycolic Acid	-
F10	Glyoxylic Acid	-
F11	D-Cellobiose	-
F12	Inosine	+
G1	Glycyl -L - Glutamic Acid	+
G2	Tricarballylic Acid	-
G3	L-Serine	-
G4	L-Threonine	-
G5	L-Alanine	-
G6	L-Alanyl - Glycine	-
G7	Acetoacetic Acid	-
G8	N-Acety l-β -D-Mannosamine	+
G9	Mono Methyl Succ inate	+
G10	Methy l Pyruvate	+
G11	D-Malic Acid	-
G12	L-Malic Acid	-
H1	Glycyl -L - Proline	+
H2	p-Hydroxy Phenyl Acetic Acid	-
H3	m-Hydroxy Phenyl Acetic Acid	-
H4	Tyramine	-
H5	D-Picose	-
H6	L-Lyxose	-
H7	Glucuronamide	-
H8	Pyruvic Acid	+
H9	L-Galactonic Acid -γ - Lactone	-
H10	D-Galacturonic Acid	-
H11	Phenylethyl-amine	-
H12	2-Aminoethanol	-

TABLE II
CARBON SOURCES UTILIZATION PROFILES FOR *ANOXYBACILLUS RUPIENSIS UNISZA-7* ON PM2A PLATE INCUBATED AT 55°C FOR 48 HOUR

Well	Substance	Results
A1	Negative Control	-
A2	Chondroitin Sulfate C	-
A3	α-Cyclodextrin	+
A4	β-Cyclodextrin	+
A5	γ-Cyclodextrin	+
A6	Dextrin	-
A7	Gelatin	-
A8	Glycogen	+
A9	Inulin	+
A10	Laminarin	+
A11	Mannan	-
A12	Pectin	-
B1	N-Acetyl-D- Galactosamine	+
B2	N-Acetyl-Neuraminic Acid	-
B3	β-D-Allose	-
B4	Amygdalin	+
B5	D-Arabinose	+
B6	D-Arabitol	-
B7	L-Arabitol	-
B8	Arbutin	+
B9	2-Deoxy-D-Ribose	-
B10	I-Erythritol	-
B11	D-Fucose	+
B12	3-D -β –D-Galacto - pyranosy l-D-	-

	Arabinose	
C1	Gentiobiose	+
C2	L-Glucose	-
C3	Lactitol	-
C4	D-Melezitose	+
C5	Maltitol	+
C6	α-Methyl –D-Glucoside	+
C7	β-Methy l-D-Galactoside	+
C8	3-Methyl Glucose	+
C9	β-Methy l-D-Glucuronic Acid	+
C10	α-Methyl –D-Mannoside	+
C11	β-Methy l-D-Xyloside	+
C12	Palatinose	+
D1	D-Raffinose	+
D2	Salicin	-
D3	Sedoheptulosan	-
D4	L-Sorbose	+
D5	Stachyose	+
D6	D-Tagatose	+
D7	Turanose	+
D8	Xylitol	+
D9	N-Acety l-D-Glucosaminitol	-
D10	γ –Amino Butyric Acid	-
D11	δ-Amino Valeric Acid	-
D12	Butyric Acid	+
E1	Capric Acid	-
E2	Caproic Acid	-
E3	Citraconic Acid	-
E4	Citramalic Ac id	-
E5	D-Glucosamine	+
E6	2-Hydroxy Benzoic Acid	-
E7	4-Hydroxy Benzoic Acid	-
E8	β-Hydroxy Butyr ic Acid	+
E9	γ-Hydroxy Butyr ic Acid	+
E10	α-Keto Valeric Acid	+
E11	Itaconic Acid	-
E12	5-Keto-D-Gluconic Acid	-
F1	D-Lac tic Acid Methyl Ester	+
F2	Malonic Acid	+
F3	Melibionic Acid	+
F4	Oxalic Acid	-
F5	Oxalomalic Acid	-
F6	Quinic Acid	+
F7	D-Ribono -1,4- Lactone	-
F8	Sebacic Acid	-
F9	Sorbic Acid	-
F10	Succinamic Acid	+
F11	D-Tartaric Acid	+
F12	L-Tartaric Acid	+
G1	Acetamide	+
G2	L-Alaninamide	-
G3	N-Acetyl-L-Glutamic Acid	-
G4	L-Arginine	+
G5	Glycine	-
G6	L-Histidine	-
G7	L-Homoserine	-
G8	Hydroxy-L-Proline	-
G9	L- Isoleucine	+
G10	L-Leucine	+
G11	L-Lysine	+
G12	L-Methionine	+
H1	L-Ornithine	-
H2	LPhenylalanine	-
H3	L-Pyroglutamic Acid	+
H4	L-Valine	+
H5	D,L-Carnitine	+
H6	Sec -Butylamine	-
H7	D,L-Octopamine	-
H8	Putrescine	-
H9	Dihydroxy Acetone	-
H10	2,3 -Butanediol	+
H11	2,3 -Butanone	-
H12	3-Hydroxy 2 -Butanone	-

ACKNOWLEDGMENT

The authors acknowledge Universiti Sultan Zainal Abidin (UNiSZA) for the financial support of this project.

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