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PARTIAL PURIFICATION AND CHARACTERIZATION OF L-ARABINOSE ISOMERASE PRODUCED FROM LOCAL ISOLATE OF *BACILLUS SUBTILIS* AH1

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ABSTRACT : Fifteen of local isolates of *Bacillus* sp. were obtained among forty isolates from different location of Iraqi soil. These isolate were subjected to primary and secondary screening and their ability for L-arabinose isomerase production were tested. The isolate A3 was the the highest in enzyme production with enzyme activity 30.53 unit/ml. The morphological and biochemical tests for isolate A3 revealed that this isolate belong to *Bacillus subtilis* and signed as AH1. Crude enzyme of L-arabinose isomerasefrom *Bacillus subtilis* AH1 was produced under optimum condition by submerged culture and partially purified by two steps including, concentration with 60% saturation of ammonium sulfate and dialysis. The results revealed that the purification folds was 1.54 and yield were 24.20%. The optimium pH for enzyme activity were 7.5 and the optimum pH of stability between 7-8 , while the optimum temperature for enzyme activity was 50°C and the optimum temperature for stability between30 -50°C for 30 min of incubation .

Key words : L-arabinose isomerase, purification, characterization, *Bacillus subtilis*, Tagatose.

INTRODUCTION

Bacillus genes one of older bacterial geneses and belong to *Bacilliaceae*. This genes was discovered by Cohn (1872). Their species were gram positive and endospore forming. *Bacillus subtilis* was considerdnon pathogenic bacteria for human, animal and plant. This bacteria was produced many of proteases and carbohydrate lytic enzyme, which used in many of industrial, medical and food applications (Schalmey *et al*, 2004). Many studies indicate that *Bacillus subtilis* and another microbial source can produce L-arabinose isomerase (Jougenson *et al*, 2004; Kim and Oh, 2005). L-arabinose isomerase can convert L-arabinose to L-ribulose (*in vivo*) by pentose –phosphate pathway, while it convert D- galactose to D- tagatose (*in vitro*) by isomerization (Jin-Ha Kim *et al*, 2010) (Fig. 1).

Because of importance of L-arabinose isomerase to produce tagatose the researcher were studied production of this enzyme from microbial sources. Many researcher indicate that during lactose metabolism in bacteria, tagatosewill be produced from galactose by L-arabinose isomerase (Ibrahim and Spradine, 1993). Tagatose was found in sterilized cow milk and dried milk in low concentrations 2-20 ppm and 800ppm respectively (Troyano *et al*, 1991). While in yoghurt 29 ppm (Saunders, 1998) and it produce as by product from lactulose syrup production and contain 0.7-1% of tagatose (Zehner *et al*, 1994). Tagatose sweetness equal 92% of sucrose sweetness (Tomomi, 2012) and consider one of low energy sugars because, it give half calories that produced from sucrose (Deok–Kun, 2007). Tagatose was used in food processing and classified by FAD as GRAS (Gilbert, 2002) and dosn't elevate glucose level in blood because it metabolized in different way that sucrose metabolized. Tagatose dosen't have effect on diabetes, it mean has not laxative effect and glycemic index was not elevate (Chiu, 2011). Numbers of researchers can purified L-arabinose isomerase produced from many types of bacteria. Hichem *et al* (2007) can purified this enzyme produced from *Lactobacillus plantarum* NC8 by precipitation with ammonium sulfite of 65-90% followed by dialysis, the yield was 14.7% and fold of purification was 19, while Lifang *et al* (2010) can purified L-arabinose isomeraseproduced from *Acidothermus cellyulolytics* by passing the extractto ion exchange chromatography column followed by gel filtration and the yelid was 27% and fold of purification was 32.7 with enzyme activity of 19.29 U/mg.

Fig. 1 : The enzymatic reaction for conversion arabinose to ribulose (*in vivo*) and conversion of galactose to tagatose (*in vitro*) (Weisha, 2007).

MATERIALS AND METHODS

Isolation and primary screening

5 gm of different location of Iraqi soil were sieved and suspended in 100 ml of sterilized distilled water . The mixture was heating at 85°C for 15 min to kill vegetative cells. Serial dilutions with peptone water were prepared until 10^{-8}. Transfer 0.1 ml of each dilutions to petridish contain nutrient agar and spreading by L-shap . incubate at 37°C for 24 h. pick out some of single colony and sub-culturing by streekingon nutrient agar to obtain pure culture. Microscopic test were done to confirmed it's purity The isolatewere maintain on slant of nutrient agar and stored in refrigerator at 4°C.

Secondary screening

The medium used in production of L-arabinose isomerase was mentioned by Zhang *et al* (2007) with some of modifications. So galactose used instead of glucose and potassium phosphate used instead of sodium acetate. The medium contain g/L (glucose 10; yeast extract 10; monopotassium phosphate 0.2; dipotassium phosphate 0.2, magnesium sulphate 0.2; manganese sulphate 0.02; arabinose 1.5). Medium pH was calibrated to 7. The medium was distributed in 250 ml of Erlenmeyer flask and autoclaved at 121°C for 15 min. Transfer 1ml of isolate suspension contain 3×10^8 cfu/ml calculated by standard curve of McFarland method (Collee *et al*, 1996) and the production of Intracellular enzyme was performed in incubator shaker (150 rpm) at 30°C for 48 h. The medium was subjected to centrifuge at 8000 rpm for 20 min to separate biomass. Suspension was ignored and the biomass re-suspended in 300 ml flask by adding 50 ml of cetyltrimethyl ammonium bromide (CTAB) 0.1 % (Chen *et al*, 1979). The enzyme was extracted in incubator shaker with 150 rpm at 30°C for 24 h. The extraction was subjected to centrifugation 10000 rpm for 15 min at 4°C. The precipitate was ignored and the supernatant was considered as crud enzyme .

Detemination of enzyme activity

Enzyme activity was determined by adding 0.1 ml of crude enzyme to 0.5ml of 0.1 M of potassium phosphate buffer and 0.2 ml of 0.5 M of galactose as substrate. The mixture was incubated in water bath at 50°C for 30 min. The reaction was stopped by adding 1 ml of 0.5 M of perchloric acid (Zhang *et al*, 2007). Tagatose can be detected by adding 0.2 ml of 1.5% cysteine – Hydrochloride and 6 ml of 70-75% sulfuric acid and 0.2 ml of 0.12% carbazole. The solution was mixed by vortex and absorbance was measured at 560 nm (Dische and Borenfrenud, 1951). Blank was done by same procedure excluded the addition of crude enzyme after stopping the reaction by adding 1 ml of 0.5 M perchloric acid. The amount of liberated tagatose was determined according standard curve for tagatose solution. The enzyme activity was defined as the amount of enzyme that liberate one micromole of tagatose in one minute under experiment conditions.

Determination of protein concentration

Protein concentration in enzyme extracts were determined by Brad Ford method (Bard Ford, 1976) and using standard curve of protein for Bovineserum albumin.

Identification of isolate produced L-arabinose isomerase

The selected isolate that can be able to produce L-arabinose isomerase was cultured on nutrient agar and incubate at 37° C for 24 h. Growth characteristic was studied (shape , size, color and colony edge) (Atlas *et al*, 1995). Microscopic assay (gram stain, cell shape, type and location of spore) were performed .Biochemical assay was achieved including oxidase test, catalase test, Vogesproskaure and Citrate utilization (MacFaddin, 2000), Starch hydrolysis (Atlas *et al*, 1995), Gelatin liquefaction (Cruickshank *et al*, 1975), Sugar fermentation (Forbes *et al*, 2007), Casien hydrolysis, Aerobic growth and growth at 7% NaCl, growth at 50°C and 60°C (Buchanan and Gibbons, 1974), Motility (MacFaddin, 2000).

Enzyme partial purification

a. Precipitation by ammonium sulphate : Crude enzyme was precipitate using ammonium sulfate with 30-60% of saturation. Adding solid ammonium sulfite to crud enzyme with continuous stirring at 4°C. Initially the ammonium sulfite was add to 30% followed by centrifuge at 10000rpm at 4°C. Precipitate was separated, while the ammonium sulphate was add to supernatant to be 60%. Then centrifugation was done again at same previous conditions. The supernatant was separated and the precipitate was dissolvedwith 0.1 M phosphate buffer pH7.5. Volume of enzyme extract , protein and enzyme activity for supernatant and precipitate in all stages ofprecipitation were determined.

b. Dialysis : Enzyme extract from previous stage was subjected to dialysis against 0.1 M potassium buffer pH

7.5 using dialysis tube with molecular weight cut-off (MWCO) 14000 at 4°C for 24 h and dialysis solution was substituted three times .

Enzme characterization

a. Optimum pH for activity and stability : L-arabinose isomerase activity was determined indifferent pH values (5-9.5). Differences between each treatment equal 0.5 using following buffers :0.1 M Acetate buffer with pH (4-5.5); 0.1 M phosphate buffer with pH (6 - 8) and 0.1 M Tris- HCl buffer with pH (8.5 - 9.5). Enzyme activity was determined (Zhang *et al*, 2007 and Dische and Borenfrenud, 1951). Optimum pH for stability was determined by incubate the crude enzyme in previous buffers for 30 min in room temperature. The residual enzyme activity was determined.

b. Optimum temperature for activity and stability : The enzyme activity was determined at 30-70°C in water bath with difference equal 10°C between each treatment at optimum pH for activity. While optimum temperature for stability was determined by incubation the crude enzyme at different temperature between 30-70°C in water bath for 30 min. Enzyme activity was determined as mentioned before.

RESULTS AND DISCUSSION

a. Isolation and identification

15 of local 1solate of *Bacillus* among 40 isolate can be obtained from different location of Iraqi soli. These isolate formed creamy to brown colony and sticky with irregular edge. Some times they are slightly elevated from agar surface when growth on agar at 37°C after 24 h. Subcultring a single colony by streeking on nutrient agar and incubated at 37°C for 24 h to obtain pure colony. Picked out some of single colony and subjected to secondary screening on liquid medium contain galactose as carbon source and arabinose as inducer for L-arabinose isomerase production by submerged fermentation at 30°C for 48 h. The biomass was separated and crud enzyme was extracted and enzyme activity was determined. The results of secondary screening revealed that the isolate A3 give high enzyme activity (30.53 U/ml) in comparison with other isolates (0-14.41 U/ml) (Fig. 2).

The isolate A3 was submitted to morphological and culturing tests, moreover biochemical tests. The Morphological test revealed that the isolate A3 was rod, Motley, G+, spore former in cell center or at sub – terminal of cell (Table 1).

Biochemical test results for Isolate A3 is summarized in Table 2.

In comparison of morophological and biochemical tests of isolate A3 with references for bacterial classification as well as Cowan and Steel's (2003), Logan and De Vos (2009), Sevdlaina and Lubka (2010). The results revealed that these characterization of isolate A3 were similar to those in *Bacillus subtilis* and designated as AH1.

b. Partial purification of Enzyme

The results show that the enzyme specific activity was increased and became 56.97mg/ml, 64.90 mg/ml after precipitation of crude enzyme by ammonium sulfite and dialysis, respectively. The fold of purification also increased and became 1.35 and 1.54 respectively also. While the yield were decreased to 31.47% after precipitation with ammonium sulfite and 24.21% after dialysis (Table 3).

The results revealed that the precipitation process by ammonium sulfite due to reduce the solubility of protein. So it precipitate by equalization the charges on protein surface and make disruption in water layer surrounded of protein and reduce it's degree of hydration (Englard and Seifter, 1990). This effect invert on increasing specific activity and fold of purification . In dialysis step the protein concentration was reduced to half because more of proteins with less than 14000 dalton were dialyized. More ever more of salts were removed from enzyme extract leading to increasing the fold of purification as indicator of purity (Segal, 1974). These results were agree with results of Kim *et al* (2006), which indicate that the fold of purification were increased to 2.2 and yield were reduced to 59 % after precipitation crud enzyme produced from *Geobacillus sterothemophilus* by ammonium sulfate. Moez *et al* (2011) can purified L-arabinose isomerase produced from *Shewanell* sp. ANA-3 by precipitation with ammonium sulfate, dialysis and gel filteration. The specific activity were increased from 26 to 164 u/mg, while Zhang *et al* (2007) can purified L-arabinose isomerase produced from *Lactobacillus plantarum* by precipitation with ammonium sulfite, dialysis, ion exchange and gel filteration. So the fold of purification were increased to 1.6 and the specific activity were increased from 12 to 19.5 U/mg, while the yield was reduced to 79%.

This variable between these studies may be led to differences with source of enzyme, moreover the differences between purification technique used in these studies.

Enzyme characterization

a. Optimum pH for activity

The results shown in Fig. 3 that L-arabinose isomerase has high activity in pH between 7 - 8 and the

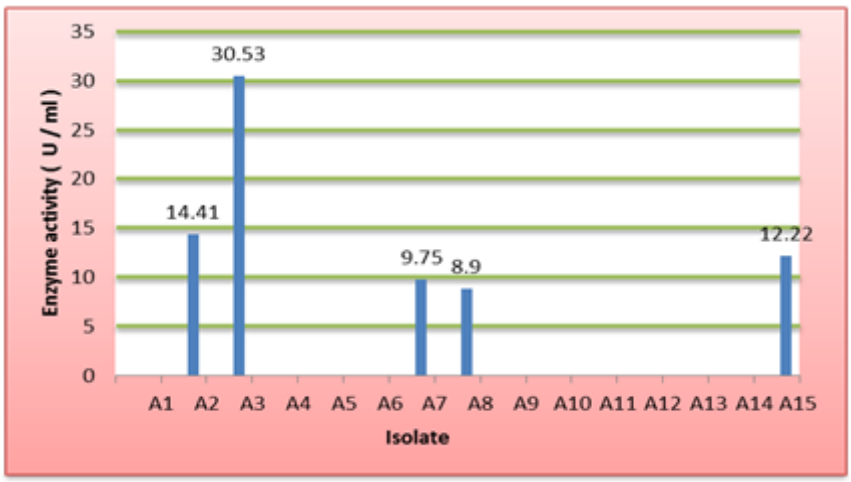

Fig. 2 : Productivity of some Isolates produced L-arabinose isomerase as enzyme activity (U/ml).

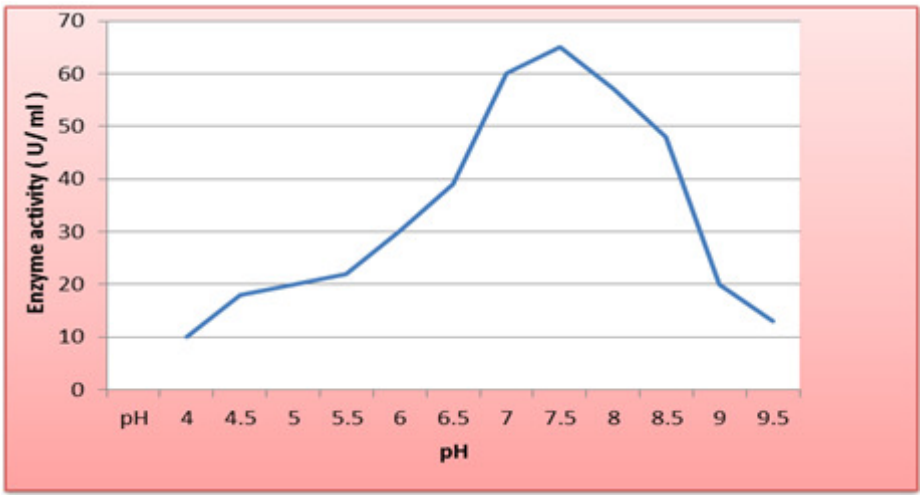

Fig. 3 : Optimum pH of activity for L-arabinose isomerase produced by local isolate of *Bacillus subtilis* AH1.

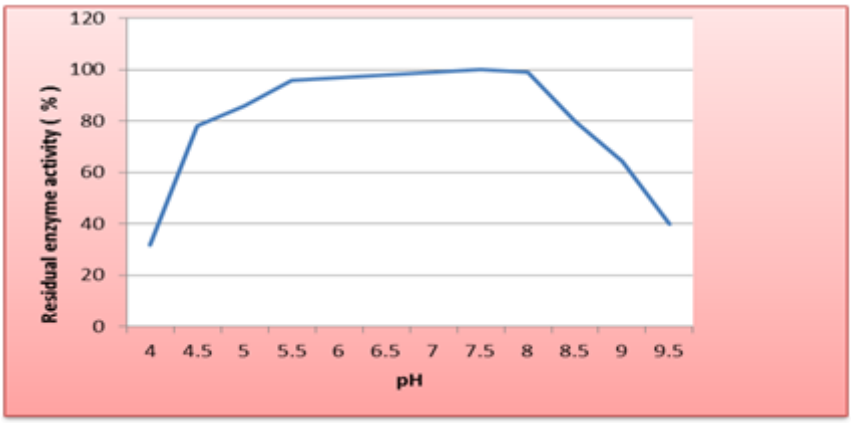

Fig. 4 : Optimum pH of stability for L- arabinose isomerase produced from local isolate of *Bacillus subtilis* AH1.

optimum pH for activity was at 7.5, the enzyme activity was 65.09 U/ml. Graduated reduction in activity in other values of pH may be due to the effect of one or more of ionic groups in active site of enzyme or substrate or both followed by changing the ionic state of these groups and the reaction between enzyme and substrate was effected.

These results were agree with Ponnandy *et al* (2008), Jin–Ha Kim *et al* (2010) that the optimum pH for activity of L-arabinose isomerase were 7.5, while Seo (2013) indicate that the optimium pH for L-arabinose isomerase activity produced from *Bacillus thermoglucosidasius* was 7.

b. Optimum pH for stability

pH stability consider as one of important characteristics for choosen the purification and storage method of enzyme.

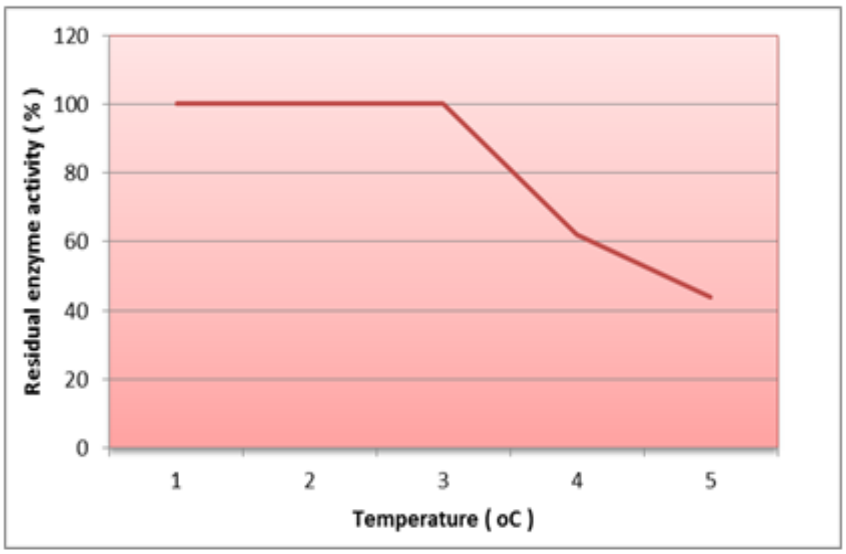

Fig. 5 : Optimum temperature of activity for L-arabinose isomerase produced by local isolate of *Bacillus subtilis* AH1.

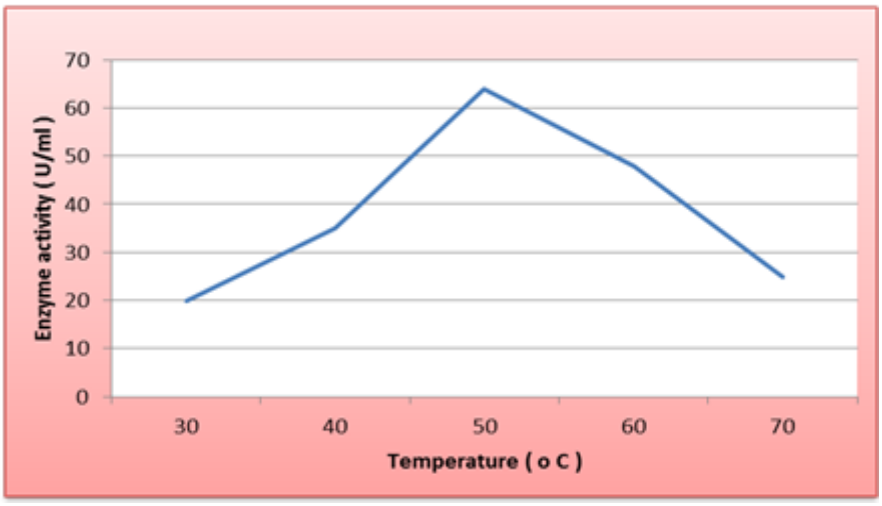

Fig. 4 : Optimum temperature curve of stability for L- arabinose isomerase produced from local isolate of *Bacillus subtilis* AH1.

Table 1 : Cultural and morphological characterization of isolate A3 produced L-arabinose isomerase.

Cultural characterization	
Growth at solid medium	Widespread and plane
Growth at liquid medium	Aerobic or facultative aerobic growth at surface or sub surface
Colony color	Creamy – brown
Colony edge	Irregular circular
Colony surface	Smooth
Morphological characteristics	
Cell shape	Long – medium bacilli
Cell dimension	2.5 × 10 µm - 0.5 × 12 µm
Motility	+
Gram stain	+
Spore shape	Helical
Spore location	Central or sub terminal

The results were indicate that the partially purified of L- arabinose isomerase produced from *Bacillus subtilis* AH1 has stability in pH value between 7-8 because, it preserved about all activity in this range, but it loss 68% of it's activity at pH 4 and preserved about 64.33% of it's activity at pH 9 (Fig. 4). The reduction of enzyme activity at acidic and alkaline values due to changes that occur in secondary and tertiary structure of enzyme , moreover ionic changes in active site that effect on pH stability of enzyme (Segal, 1976). The studies were differ in determine the optimum pH of stability for L-arabinose isomerase. Hichem *et al* (2007) indicate that L- arabinose isomerase produced from *Lactobacills plantarum* NC8 was stable in acidic pH and preserved about 89% of it's activity at pH 5, while Marta and Jozef (2012) indicate that enzyme produced from *Arthrobacter* sp. 22c has high stability at pH 7 and the enzyme was preserved about 90% of it's activity after incubation for 72 h.

Tabl 2 : Biochemical tests of isolate A3.

Test	Result	
Oxidase	+	
Catalse	+	
Growth at 50 ° C	+	
Growth at 60 ° C	-	
Vogesproskauer	+	
Starch hydrolysis	+	
Casien hydrolysis	+	
Reduction of citrate	+	
Liquefaction of gelatin	+	
Growth in anerobic condition	-	
Growth at 7 % of NaCl	+	
Litmus milk	+	
Sugar Fermentation and gas production		
Type of sugar	**Acid production**	**Gas production**
Xylose	+	-
Glucose	+	-
Arabinose	+	-
M annitol	+	-
Lactose	-	-
Sucrose	+	-
Maltose	+	-

c. Optimum temperature for activity

The optimum temperature of enzyme activity of L-arabinose isomerase from local isolate of *Bacillus subtilis* AH1 was increased at more than 40°C and it's maximum value was 64.03 U/ml at 50°C (Fig. 5). This increasing in enzyme activity with increasing of temperature due to risingof movement energy for enzyme and substrate particles, subsequently increasing of reaction between enzyme and substrate (Segal, 1976). The enzyme activity was reduced at minimum value 24. 80 U/ml at 70°C. This reduction due of changing in tertiary structure of enzyme as result of temperature absorption by enzyme, subsequently due to denaturation of enzyme and losing of it's activity.

These results were agree with Ponnandy (2008) that indicate the optimum temperature of L-arabinose isomerase activity from *Bacillus licheniformis* was 50°C, while Jorgenson *et al* (2004) indicate that optimum temperature for enzyme produced from *Thermoanaerobacter mathranii* was 65°C. Also, Min *et al* (2012) was indicate that the optimum temperature of free and immobilized L-arabinose isomeras were 60°C and 75°C, respectively.

d. Optimum temperature for stability

Temperature stability was one of important factor to determine the temperature degree that the enzyme will be preserved it's activity in application field. The results indicate that the L-arabinose isomerase produced from local isolate of *Bacillus subtilis* AH1 was stable when incubate at 30-50°C for 30 min and preserved about 62% of it's activity at 60°C and loss about 52% of it's activity at 70°C under same condition (Fig. 6).

The losing of enzyme activity with increasing of temperature during incubation due to denaturation and changing of tertiary structure of enzyme which effect on enzyme reaction with substrate. The studies differ in determination the temperature stability of L-arabinose isomerase. Moez *et al* (2010) indicate that the enzyme has high stability at 35°C when incubated for 24 h and preserved about 90% of it's stability at 40°C, while Marta and Jozef (2012) indicate that the enzyme has high stability at 50°C. Thisvariation due tothe differences in source of enzyme extract, moreover differences between conditions of bacterial growthand enzyme production.

Table 3 : Purification steps of L-arabinose arabinose produced by locally isolate of Bacillus *subtilis* AH1.

Purification step	Volum (ml)	Protein (mg / ml)	Specific activity (U/ mg)	Total activity (U)	Fold of purification	Yield %
Crude enzyme	170	1.26	42.07	9011.7	1	100
Precipitation by ammonium sulfite 30-60%	60	0.83	56.97	2836.8	1.35	31.47
Dialysis	70	0.48	64.90	2181.91	1.54	24.21

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