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Improvement of uricase production from *Bacillus subtilis* RNZ-79 by solid state fermentation of shrimp shell wastes

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Abstract: Among various strains of *Bacillus subtilis*, the strain RNZ-79 was selected for this study due to the highest uricase productivity in solid state cultures containing shrimp shell wastes, highest V_{max} (0.42 μM $mg^{-1}min^{-1}$) and lowest value of K_m (56 μM). Maximum productivity was observed at pH 7.6 and 45 °C with an inducer concentration of 0.4% (w/v) uric acid and moisture content of 80% (v/w). An inoculum of 7% (v/v) and particle size of 354–500 μm for substrate were optimal for productivity after 54 h of fermentation. None of the tested carbon and inorganic nitrogen sources had stimulatory effect on uricase productivity. KH_2PO_4 at 0.12% (w/v) was the best source of phosphorus. Final uricase productivity was 5.05-fold more than the initial productivity. Enzyme purification increased the specific activity to 25-fold with a recovery of 36%. Furthermore, the purified enzyme showed a molecular mass of 33.7 kDa. Purified uricase was optimally active at pH 8.0 and 40 °C and maximally stable at pH 7.0-10.0 and till 70 °C for 30 min. The half-life ($t_{1/2}$) at 60, 70, 80, 90 and 100 °C were 87.0, 56.6, 30.0, 24.2 and 15.6 min, respectively, and the calculated midpoint temperature (T_m) was 66.85 °C. Interestingly, purified enzyme exhibited a good storage stability for 3 months and none of uric acid analogues were competitive inhibitor, indicating a high specificity of uricase.

Key words: *Bacillus subtilis*; enzyme characterization; optimization of production; protein purification; solid state fermentation; uricase.

Abbreviations: CB, corn bran; LSCs, liquid state cultures; LSF, liquid state fermentation; RB, rice bran; SBP, soybean powder; SSCs, solid state cultures; SSF, solid state fermentation; SSP, shrimp shell powder; WB, wheat bran.

Introduction

The purine degradation pathways lead to uric acid as a common product. Further degradation can result in different final products, from allantoin to ammonia and CO_2, the latter being the most frequent product. Humans lack uricase system, the formed uric acid is thus excreted out of the body through urine (Werner & Witte 2011). Under unbalanced situations, uric acid is accumulated in blood over the standard value, leading to some diseases, like gout, idiopathic calcium urate nephrolithiasis and renal failure (Nakagawa et al. 2006; Nanda & Babu 2014). Treatment of such conditions requires the intake of exogenous uricase as a protein drug, like Rasburicase® .

Uricase or urate oxidoreductase (EC 1.7.3.3) catalyses the oxidation of purine ring of uric acid to a well water-soluble cyclic allantoin. It can be obtained from different sources, such as animals (Wallrath & Friedman 1991), fungi (Yazdi et al. 2006), plants (Montalbini et al. 1999), yeasts (Adámek et al. 1990) and bacteria (Yamamoto et al. 1996; Nanda & Babu 2014).

Uricases have furthermore a beneficial use in the enzymatic evaluation of uric acid concentration in urine and blood for the diagnosis of gout disease (Kai et al. 2008). The concentration of uric acid can be estimated by measuring the amount of hydrogen peroxide formed with the 5-hydroxyisourate as the two end products of the relevant enzyme-substrate (i.e. uricase – uric acid) reaction.

Solid state fermentation (SSF) is an alternative cultivation system for the production of microbial products. It has several advantages over the classical liquid state fermentation (LSF). These include absence of rigorous control of fermentation parameters, simpler fermentation media, simple fermentation equipments, lesser fermentation space, easier aeration, less effluent generation, higher concentration of products and they provide conditions closer to natural habitats of microorganisms (Pandey et al. 1999). Fungi have been widely utilized in SSF for production of enzymes and other biomaterials, while bacteria have rarely been utilized in SSF (Lonsane et al. 1992; Pandey et al. 2007).

In the present paper, we report the production of a promising uricase from *Bacillus subtilis* strain RNZ-79 under SSF. The RNZ-79 uricase showed high thermal

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stability, pH stability, storage stability and high substrate specificity towards uric acid suggesting its potential for routine clinical application in measuring uric acid levels in body fluids.

Material and methods

Materials

Shrimp shells were obtained from the Central Laboratory for Aquaculture Research, present in Abbassa, Sharkia, Egypt. In the preparation of shrimp shell powder (SSP), the shells were washed keenly with tap water, dried, milled and sieved to different diameters for using in conjunction with uric acid as main carbon and nitrogen source for uricase biosynthesis. Sephadex G-75 FF and DEAE-Cellulose were purchased from Amersham Pharmacia Biotec (Sweden). Xanthine-agarose, uric acid and uric acid analoguess (allopurinol, oxypurinol, xanthine and hypoxanthine) were from Sigma Chemical Company. Protein standards for SDS-PAGE were purchased from Invitrogen (Shanghai, China). Other chemicals employed in this study were of analytical grade.

Microorganisms and cultivation conditions

Different strains of*B. subtilis* from the culture collection of the Laboratory of Bacteriology, Department of Microbiology, Zagazig University, Zagazig, Egypt, were employed for this study for uricase production by SSF technique. Bacterial identification was confirmed based on the phenotypic and biochemical characteristics outlined in the Bergey's Manual of Systematic Bacteriology. Strains were maintained in 50% glycerol at –15 °C.

Uricase productivity by selected strains was tested onto urate clearing medium containing (in g/L): bacteriological agar, 15; yeast extract, 3; glucose, 2; and uric acid, 2; at pH 7.0. Dishes were then incubated for 72 h at 35 °C. A clear zone around bacterial growth gave a confirmation of uric acid degradation.

Medium preparation and cultivation conditions

Solid substrates [rice bran (RB), wheat bran (WB), corn bran (CB), SSP and soybean powder (SBP)] were sundried, crushed and used as substrates. Solid state cultures (SSCs) were prepared in 250 mL Erlenmeyer flasks each containing 20 g powder of the solid substrate and 25 mL of mineral medium. The mineral medium contained (in g/L): uric acid, 2.0; $MgSO_4.7H_2O$, 5; KH_2PO_4, 0.8; $CaCl_2$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot 7H_2O$, 0.05; and $ZnSO_4 \cdot 7H_2O$, 0.02; with pH adjusted at 7.0. The flasks were then autoclaved at 121 °C for 30 min, then cooled and inoculated with 1% (v/v) bacterial suspension (3×10^8 cells/mL). Screw cap Erlenmeyer flasks were used to avoid water loss by evaporation. Unless otherwise stated, culture incubation was done at 40 °C for 72 h.

On the other hand, liquid state cultures (LSCs) were prepared by suspending 1 g of solid substrate in 50 mL of mineral medium in 250 mL Erlenmeyer flasks and sterilized at 121 °C for 15 min. Each flask was seeded with 1 mL bacterial suspension (3×10^8 cells/mL) and shake-incubated at 200 rpm for 72 h at 40 °C.

Enzyme assay and protein measurement

Uricase activity was measured according to Adámek et al. (1989) by lowering in absorbance at 293 nm. To 2 mL of uric acid solution containing 10 µg/mL of 0.1 M borate buffer (pH 8.5) 1 mL of enzyme was added. Incubation was done for 10 min at 35 °C and terminated by 0.2 mL of 0.1 M KCN solution. In the blank sample, the KCN solution was added to the mixture prior the addition of enzyme. Absorbance of both samples was measured at 293 nm and the difference of reading was equivalent to the decrease of uric acid concentration during reaction. One unit of the uricase activity is equal to enzyme concentration, which converts 1 µmole of uric acid to allantoin per min under standard assay conditions. The protein concentration was measured according to Lowry et al. (1951).

Enzyme extraction

At the end of fermentation period, uricase was extracted from the fermentation solids by addition of 0.1 M phosphate buffer at pH 7.0 (buffer A) and keeping the flasks on a rotatory shaker at 150 rpm for 1 h at room temperature. The suspension was filtered through Whatman No. 1, then centrifuged at 7,000 × *g* for 10 min. For each 1 g dry substrate taken, exactly 4 mL enzyme extract was recovered.

Enzyme purification

Uricase was purified after optimizing the major physical and nutritional factors affecting productivity under SSF. This was done in four steps including fractional precipitation with $(NH_4)_2SO_4$, anion-exchange chromatography (DEAE-Cellulose), gel filtration chromatography (Sephadex G-75 FF) and affinity chromatography (Xanthine-agarose), respectively. All purification procedures were done at 4 °C. Exactly, 3.87 L filtrate was recovered from fermented solids, then solid $(NH_4)_2SO_4$ was added. The precipitate of 50–80% $(NH_4)_2SO_4$ fraction was collected by centrifugation (7,000 × *g* for 10 min, 4 °C). Crude enzyme was then dissolved in least volume of buffer A, and dialyzed against the same buffer until complete removal of $(NH_4)_2SO_4$. The dialyzed enzyme was then applied to DEAE-Cellulose column (3×30 cm^2) previously equilibrated with buffer A and then eluted with buffer A in a linear gradient of 0–1 M KCl (flow rate: 2 mL/min). Fractions with uricase activity were pooled and lyophilized. The obtained uricase was subsequently dissolved in least volume of buffer A, dialyzed with the same buffer containing 0.1 M KCl and applied to Sephadex G-75 column (3×50 cm^2) equilibrated with the same buffer. Crude uricase was then eluted with the same buffer (flow rate: 2 mL/min) and fractions with the highest activity were pooled and applied to Xanthine-agarose column (1×5 cm^2) previously equilibrated with 20 mM phosphate buffer (pH 7.8) with 2 mM 2-mercaptoethanol and 0.5 mM ethylenediaminetetraacetic acid (buffer B) at a flow rate of 10 mL/h. After enzyme adsorption, the column was washed with 15 mL of buffer B followed by 10 mL of 20 mM KCl in the same buffer. Enzyme was then eluted specifically with 0.5 mM uric acid in the same buffer (Alamillo et al. 1991). Active fractions were lyophilized and then the SDS-PAGE was carried out to determine molecular mass and to ensure homogeneity of enzyme. SDS-PAGE was performed as described by Laemmli (1970), using 5 and 15% (w/v) stacking and separating gels, respectively.

Biochemical characteristics of purified enzyme

The influence of the pH on uricase activity was studied by incubating the enzyme in three buffer systems (0.1 M citrate for pH 5.0–6.0, 0.1 M phosphate for pH 6.0–8.5 and 0.1 M borate for pH 8.5–13.0) with uric acid as substrate at 35 °C. The pH stability of the purified uricase was tested at the same range by pre-incubating enzyme solution for 30 min at 35 °C without substrate. At the end of incubation, remaining activity was measured at pH 8.0 and 35 °C.

Table 1. Uricolytic activity of selected strains of *B. subtilis* in liquid and solid state cultures containing different solid substrates.[a]

Strain	Uricolytic activity (mU/mL)									
	Wheat bran		Rice bran		Corn bran		Soybean		Shrimp shell	
	LSF	SSF	LSF	SSF	LSF	SSF	LSF	SSF	LSF	SSF
B. subtilis K-22	121	134	99	141	74	181	166	235	182	221
B. subtilis K-42	84	95	72	73	95	110	146	179	131	81
B. subtilis RNZ-79	192	245	176	226	144	209	207	288	204	323
B. subtilis SCI-63	51	83	22	62	57	39	102	143	22	89
B. subtilis WSF-19	65	97	88	116	71	141	139	191	192	217
B. subtilis ZU-50	77	85	33	76	46	66	52	86	95	171

[a] LSF, liquid state fermentation; SSF, solid state fermentation.

The influence of temperature on uricase activity was studied by incubating the enzyme preparation in potassium phosphate buffer (0.1 M, pH 8.0) with uric acid as substrate at various temperatures (20–90 °C). The thermal stability was determined by subjecting the enzyme to various temperatures (60–100 °C) without substrate. After 15 to 60 min, tubes were cooled and residual activity was determined under optimized conditions. Storage stability at 4 °C and pH 8.0 was also tested. Uricase was stored for 3 months in several organic solvents (acetone, acetonitrile, ethanol, isopropanol, ethyl acetate, dimethylsulfoxide, dimethylformamide and diethyl ether) at 20% (v/v) concentration and then the remaining activity was measured at 40 °C and pH 8.0. Uric acid analogues (oxypurinol, allopurinol, xanthine and hypoxanthine) were tested at 0.1 mM concentration as alternative substrates for uricase under standard assay conditions applied for uric acid.

Statistical analysis

All experiments were performed in triplicates unless otherwise specified. The results data were presented as averages of triplicates ± SDs.

Results and discussion

Uricase is a therapeutic protein used widely to solubilize urate crystals in gout and hyperuricemia by catalysing the enzymatic oxidation of uric acid. For this, uricases from several microorganisms have been isolated. To the best of our knowledge no single report described uricase production under the SSF. *B. subtilis* is a common uricase producer (Schultz et al. 2001; Huang & Wu 2004; Pfrimer et al. 2010) and a producer of biomaterials under SSF (Krishna & Chandrasekaran 1996; Pandey et al. 1999; Baysal et al. 2003). For this, different strains of *B. subtilis* obtained from the culture collection of our laboratory were selected for this study with the aim to maximize uricase production by SSF technique.

Uricases of tested strains were of extracellular-type as there was no enzymatic activity inside bacterial cells as reflected by cell disruption. Extracellular uricases were also reported for some bacterial resources, such as *Bacillus fastidiosus* (Bongaerts et al. 1978), *Pseudomonas aeruginosa* (Saeed et al. 2004; Abd El Fattah et al. 2005),*Microbacterium* sp. ZZJ4-1 (Kai et al. 2008), *Bacillus thermocatenulatus* (Lotfy 2008) and *Bacillus cereus* strain DL3 (Nanda & Babu 2014). However, uricases of *Aspergillus flavus*, *Neurospora crassa*, *Mucor hiemalis* (Yazdi et al. 2006), *Candida albicans* and *Candida utilis* (Yokoyama et al. 1988) are intracellular, the cell disruption is thus required for enzyme extraction.

Substrate and strain selection

Table 1 shows uricase productivity by selected strains of *B. subtilis* in both LSCs and SSCs containing several nutritional solid substrates (SBP, WB, RB, CB and SSP). All investigated strains at most treatments showed higher uricase productivity in SSCs in comparison with LSCs. The superiority of SSCs over submerged types for the production of some enzymes by microorganisms may be due to their growth under conditions similar to their natural habitats (Pandey et al. 1999, 2007). Figure 1a shows the best enzyme productivities by the tested strains. Strain RNZ-79 was found to produce the highest levels of uricase in cultures containing SSP as a nutritional substrate (thus SSF increased enzyme productivity by 1.6-fold). It was followed by strain K-22 grown in SBP matrix (thus SSF increased enzyme productivity by 1.4-fold). RB and CB proved to be the least favourable for uricase production by the tested strains. This may due to much fibres and lesser minerals, carbon and nitrogen content.

Low value of Michaelis-Menten constant K_m is very important for clinical and industrial enzymes; thus measuring of the uricase K_m was especially mentioned in most reports dealing with this enzyme. To determine the kinetic parameter K_m for uricases produced from selected strains of *B. subtilis*, uric acid was used as substrate. The constant K_m was determined from the intercepts and slopes of the regression lines of the Lineweaver-Burk plot (Fig. 1b) by determining the enzymatic activity at 35 °C. The apparent $K_m$values for crude uricases from strains K-22, K42, ZU-50, SCI-63, RNZ-79 and WSF-19 were 101, 383, 186, 121, 56 and 328 μ M, respectively.

The lowest value of K_m was determined for uricase of strain RNZ-79 (56 μM) and was less than that reported for *Arthrobacter globiformis* FERM BP-360 (75 μM; Suzuki et al. 2004), *Bacillus* sp. TB-90 (75 μM; Yamamoto et al. 1996), *Microbacterium* sp. ZZJ4-1 (310 μM; Kai et al. 2008), *B. thermocatenulatus* (250 μM; Lotfy 2008) and *B. fastidious* A.T.C.C. 26904 (204 μM; Zhao et al. 2006). But it was higher

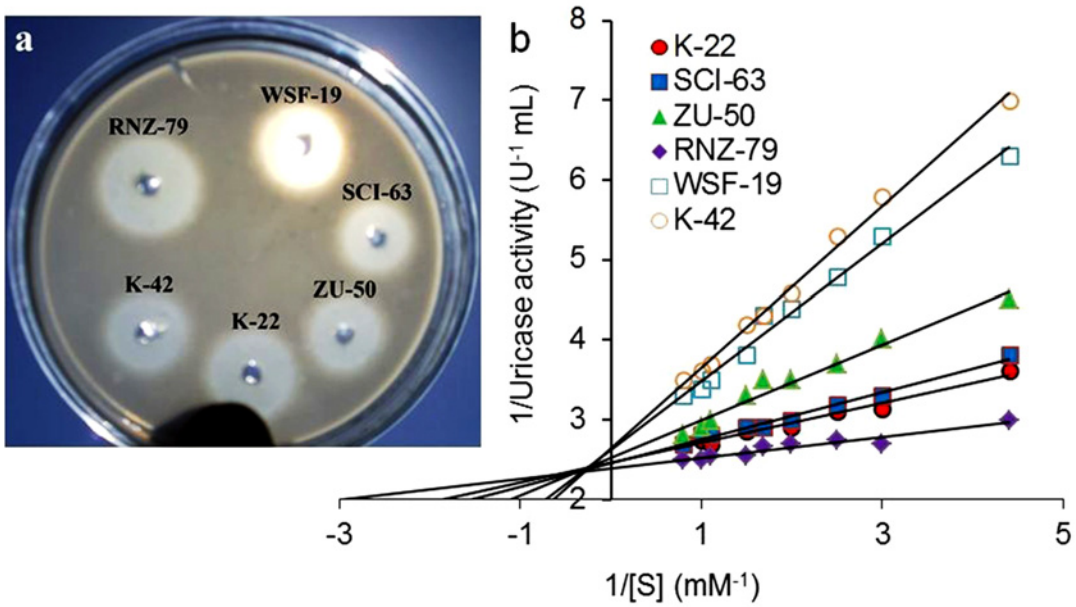

Fig. 1. (a) Urate plate showing the best activity of crude uricases of selected strains of *B. subtilis* on medium containing 0.2% uric acid and 1.5% agar with pH adjusted at 7.0 after being incubated at 35 °C for 24 h. Strains RNZ-79, WSF-19 and ZU-50 crude enzymes produced the highest uricolytic activity in medium containing SSP, while strains K-22, K-42 and SCI-63 produced the highest uricolytic activity in medium containing SBP as a solid substrate. (b) Lineweaver-Burk plot of uricases produced by tested strains of *B. subtilis*. The abscissa and ordinate indicate the reciprocal of substrate concentrations and uricase activity, respectively.

than that observed for the recombinant urate oxidase of *Bacillus* sp. TB-90 (14 μM; Hibi et al. 2014). The strain RNZ-79 was selected for further study due to the highest uricase productivity (Table 1 and Figure 1a) and the lowest K_m value (Fig. 1b).

Profile of enzyme production

There was no detectable intracellular or even extracellular uricases from the tested strain in absence of enzyme substrate (uric acid). Thus the uricase of strain RNZ-79 was a uric-acid-inducible enzyme. Initially 0.2% (w/v) of uric acid was used in culture media for enzyme induction. Inducible uricases were also reported in LSCs for *B. cereus* strain DL3 (Nanda & Babu 2014), *M. hiemalis* (Yazdi et al. 2006) and *B. thermocatenulatus* (Lotfy 2008) with best concentrations at 0.2, 0.7 and 0.3% (w/v) of uric acid, respectively.

Time course of uricase production by the strain RNZ-79 in presence of uric acid and SSP was explored (data not shown). Visual bacterial growth was observed after 12 h followed by formation of extensive wrinkled bacterial pellicle on SSP surface characteristic of *B. subtilis*, which gradually increased in density and wrinkling by time. Visual inspection indicated that bacterial cells impregnated the whole substrate within 24 h, while un-inoculated blanks showed no detectable bacterial growth. Uricase productivity was not significant during the first 24 h of fermentation, thereafter, the enzyme concentration increased reaching the maximum after 54 h, then it gradually decreased with longer fermentation periods. This result disagreed with that of LSCs of *P. aeruginosa* (36 h; Anderson & Vijayakumar 2011).

Effect of inoculum ratio

Each 1 mL inoculum was adjusted to 3×10^8 cells using 1.0 McFarland standard solution according to McFarland nephelometric method (Bailey et al. 1986). Optimization of inoculum (Fig. 2a) indicated that maximal enzyme productivity was obtained at 5–10% (v/v) (based on the volume of mineral solution), whereas the enzyme productivity was lower when inocula were beyond this range. This may be attributed to the high moisture content introduced by large inoculum sizes, while small inocula could not initiate fermentation process easily due to few number of cells. In this connection, Nanda & Babu (2014) reported 5% (v/v) inoculum for maximum uricase productivity in LSCs of *B. cereus* DL3.

Effect of moisture level

Water is used in limited volumes in SSF and it is a basic parameter for mass transfer of solutes across cell membrane and has desirable effects on the physicochemical properties of nutritional solids, which in turn affects enzyme productivity. The control of moisture level could thus be used to control and modify the metabolic activity of the fermenting microorganism (Lonsane et al. 1992). The correlation between enzyme productivity and moisture level of SSP was studied (Fig. 2b). It was found that, 60–90% (v/w) moisture level resulted in maximal uricase production after 54 h of fermentation process and out of this range, there was no further uricase productivity. Lower moisture level led to sparse growth and dry culture resulting in lower uricase productivity, while high moisture level led to decreased porosity resulting in an additional barrier for diffusion

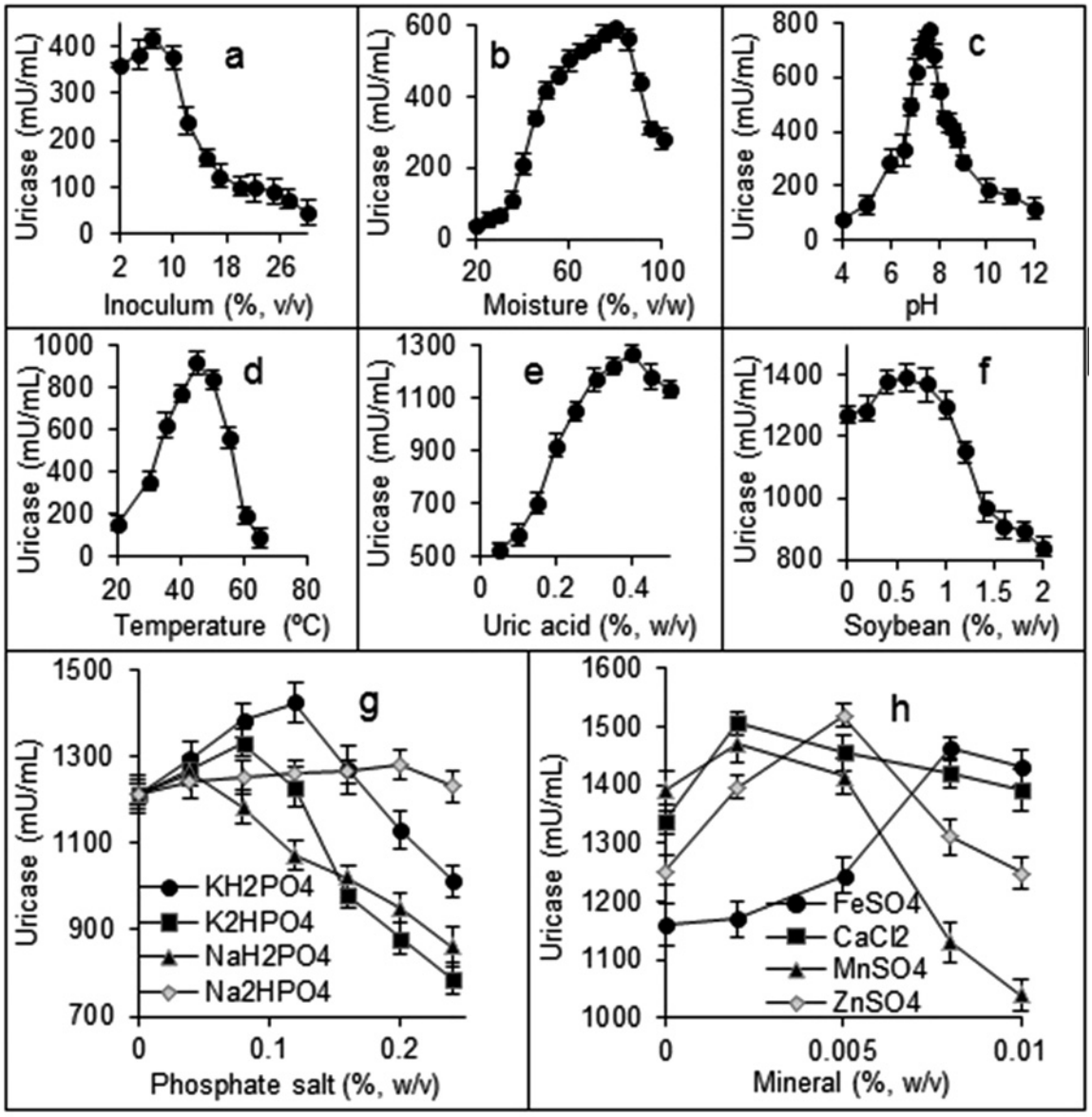

Fig. 2. Effect of inoculum size (a), moisture level (b), initial pH (c), fermenting temperature (d), uric acid concentration (e), soybean flour concentration (f), phosphorus source (g) and essential minerals (h) on uricase productivity by *B. subtilis* RNZ-79 under solid state fermentation.

of bacterial cells into the substrate. Thus, moisture level of 80% (v/w) was adapted in the following experiments. In general sense, it has been stated that filamentous fungi (20–70%) and yeast (60–70%) prefer relatively low moisture values for enzymes productivity, whereas bacteria prefer higher moisture values with solid matrices (Pandey et al. 1999, 2007).

Effect of substrate particle size

Particle size directly affects porosity and surface area of the nutritional substrate. It also affects the interchange with bacterial growth, mass and heat transfer during SSF process. In this experiment shrimp shell was air dried at room temperature, milled and sieved through American Standard Sieves No. 18, 20, 35, 45, 80, 140 and 170 to get different particle size fractions (>1000 μm, 1000–841 μm, 841–500 μm, 500-354 μm, 354–177 μm, 177–105 μm and <105 μm, respectively) of SSP matrix. Best results of uricase productivity were obtained with substrate particles of 354-500 μm (626 mU/mL), followed by 500-841 μm (586 mU/mL). The lowest productivity was obtained with substrate particles lesser than 105 μm (516 mU/mL). With significantly smaller size substrate particles, the porosity was less but the surface area was greater so that bacterial cells could not reach deep into pores and hence between particles. Visual assessments of bacterial growth showed that bacterial growth was greatly stimulated and was mainly restricted to culture surface. On the other hand, with extra larger substrate particle sizes, bacterial growth extended into interparticle voids with decreased surface growth and enzyme productivity (Pandey et al. 1999, 2007). The two opposing factors, increase in porosity and decrease in surface

area may have been compensated to approach a value corresponding to optimum bacterial growth and uricase productivity at particle size of 354–500 μm. Comparison of particle size effect with other enzymes produced by *B. subtilis* under SSF revealed that the α-amylase production by *B. subtilis* was maximally attained with banana fruit stalk particles of 400 μm (Krishna & Chandrasekaran 1996) and rice husk particles of 500 μm (Baysal et al. 2003).

Effect of pH and temperature

Results presented in Figure 2c clearly show the dependence of uricase productivity on the initial pH of the fermentation medium. The pH 7.6 was optimal for maximal uricase production from *B. subtilis* RNZ-79. The optimal uricase productivity near the neutral pH range may be due to the maximum activity of H^+-pumping ATPase, the balance of the ionic strength of plasma membrane and the optimum fluxing of ions that influence the activities of bacterial calmodulin homologs and adenylate cyclase (Swan et al. 1987). In this connection, pH 7.0 was reported for maximum uricase production by LSCs of *B. thermocatenulatus* (Lotfy 2008), *P. aeruginosa* (Anderson & Vijayakumar 2011) and *B. cereus* DL3 (Nanda & Babu 2014).

Concerning the effect of incubation temperature upon uricase productivity (Fig. 2d), 45 ℃ was optimal for attaining maximum enzyme production. This result disagreed with the results of uricase production by LSCs of *P. aeruginosa* (35 ℃; Anderson & Vijayakumar 2011) and *M. hiemalis* (30 ℃; Yazdi et al. 2006).

Effect of carbon and nitrogen augmentation

Although we used SSP and uric acid as the main C/N source in this study, we tested the effect of further C/N augmentation in order to maximize uricase productivity at the highest levels. Several carbon sources (fructose, saccharose, lactose, glucose, starch, sodium acetate and citric acid) were studied at 1% (w/v) and the results showed that none of them significantly increased uricase production of *B. subtilis* RNZ-79 in comparison with blank (presence of SSP and uric acid as the sole carbon source). Thus in all experiments, uric acid was used as an enzyme inducer and as C/N source along with SSP, and no additional carbon source was added as an augment. The effect of uric acid concentration on uricase productivity was studied. The optimal concentration was found at 0.4% (w/v), while higher concentrations of uric acid inhibited the uricase productivity (Fig. 2e).

The effects of nitrogen source, i.e. beef extract, soybean, peptone, yeast extract, sodium glutamate, casein and ammonium salts (sulphate, acetate, chloride and nitrate) on uricase productivity by *B. subtilis* RNZ-79 were also studied in comparison with blank (presence of SSP and uric acid as the sole C/N source) at 0.5 % (w/v) concentration. Results showed (data not shown) that inorganic nitrogen sources had not a stimulation effect on uricase production. The possible reason is that complex nitrogen sources contain several readily available amino acids that could be metabolized directly by cells to initiate growth and consequently promoting uricase productivity. Among the complex nitrogen sources, SBP flour was the best source. To determine the optimal value of SBP flour, several concentrations (0.2–2.0%, w/v) were tested. The optimal uricase production (1,392 mU/mL) was observed at 0.6% (w/v) with 1.1-fold higher than that of blank (Fig. 2f). In the contrary, Yazdi et al. (2006) found that, both organic and inorganic nitrogen sources did not enhance the uricase productivity of *M. hiemalis*; furthermore they used 0.7% (w/v) uric acid as enzyme inducer and nitrogen source. The maximal enzyme induction level for *C. utilis* was obtained at 0.6% (w/v) uric acid in a basal medium containing uric acid as the sole nitrogen source (Liu et al. 1994). However, in the study of Adamek et al. (1990), uricase induction was better in presence of hypoxanthine than of uric acid. In addition, *B. cereus* DL3 and *C. tropicalis* utilized 0.2 and 0.25–0.6% (w/v) of uric acid for maximum uricase production in LSF (Tanaka et al. 1977; Nanda & Babu 2014).

The dependence of uricase production on further nitrogen augmentation and the independence on further carbon augmentation may be attributed to the fact that nitrogen limiting conditions may lead to an overall drop in the cell energy pool due to the consumption of intracellular ATP and GTP via the purine degradation and salvage pathways (Abd El Fattah et al. 2005). Another reason comes from the available sequence data for *B. subtilis* genome for the potential involvement of catabolite repression protein during uricase productivity and that *B. subtilis* purine degradation operon (*puc*) is directly operated by at least three general regulators including TnrA, a regulatory protein that is involved in the activation of nitrogen-regulated genes in response to nitrogen limiting growth conditions (Brandenburg et al. 2002).

Effect of phosphorus source

Phosphorus belongs to the crucial elements controlling signal transduction, integral structure, viability and cellular reactions of microbial cells, which in turn affects enzyme productivity. Data shown in Figure 2g show the variation of phosphorus sources on uricase production from tested strain. The maximum enzyme productivity (1,423 mU/mL) was obtained using 1.2 g/L KH_2PO_4, followed by 0.8% g/L K_2HPO_4. On the other hand, uricase productivity was relatively decreased in the presence of NaH_2PO_4 and Na_2HPO_4. Similar result was obtained for uricase production by LSCs of *P. aeruginosa* (Abd El Fattah et al. 2005). The preferential use of potassium dihydrogen phosphate (KH_2PO_4) could be explained on the basis of the fact that potassium as monovalent cation (K^+) at lower number of atoms may be better to enhance membrane depolarization and cellular transport across bacterial cells.

Effect of minerals

Minerals greatly affect enzyme production as they are utilized by bacteria in the biosynthesis of metaloen-

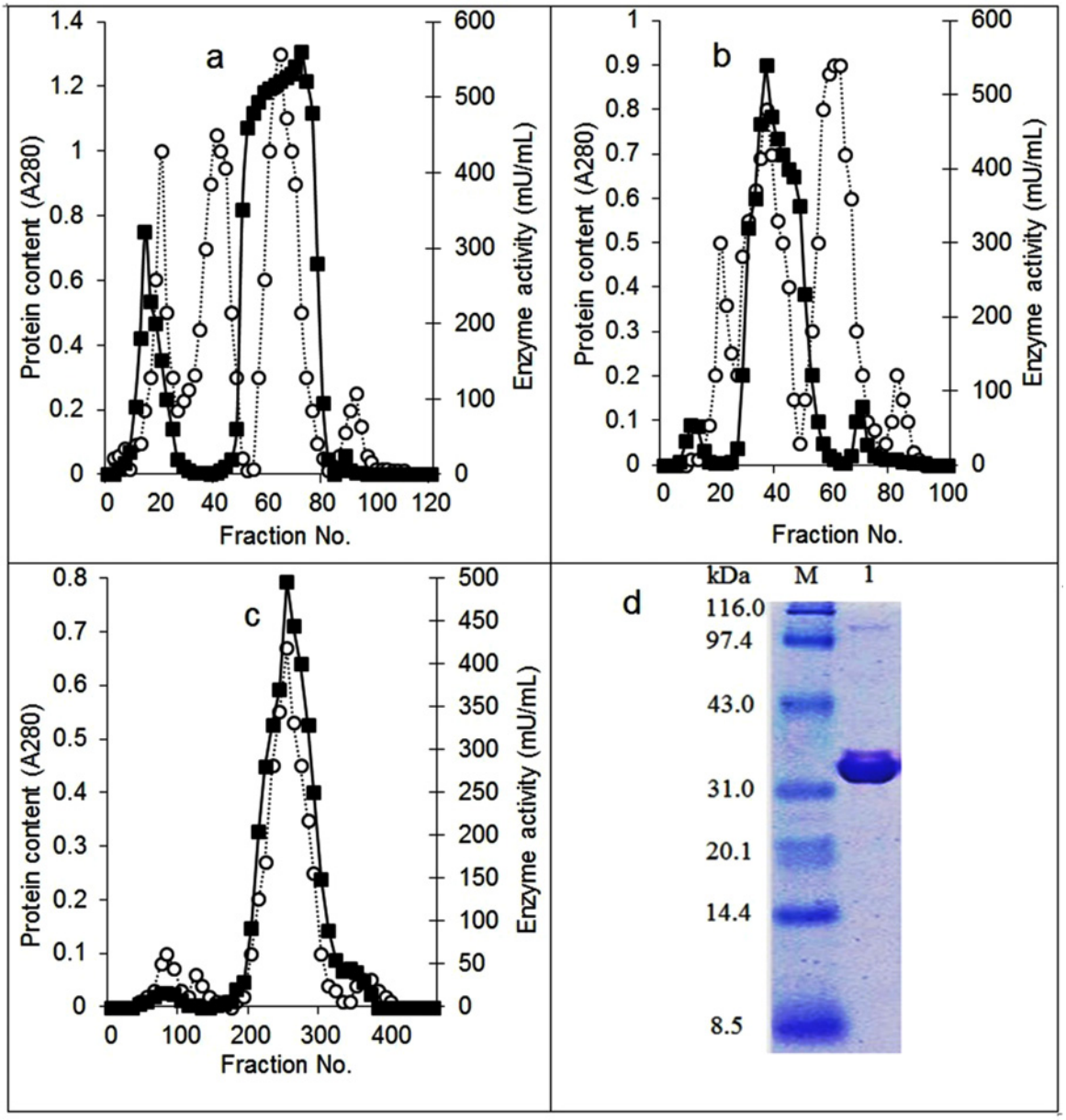

Fig. 3. Elution profile of uricase enzyme on DEAE-cellulose (a), Sephadex G-75 FF (b) and xanthine-agarose (c). The empty curcles and filled squares represent protein content (A_{280}) and enzyme activity (mU/mL), respectively. The DEAE-cellulose and Sephadex G-75 columns were eluted with phosphate buffer with a linear gradient of 0-1 M KCl and 0.1 M KCl, respectively, whereas the xanthine-agarose column was previously equilibrated with phosphate buffer containing 0.5 mM ethylenediaminetetraacetic acid and 2 mM 2-mercaptoethanol (buffer B). After enzyme adsorption, the column was washed with 15 mL of the buffer B followed by 10 mL of 20 mM KCl in the buffer. Uricase was then eluted with 0.5 mM urate in the same buffer. (d) SDS-PAGE pattern of uricase. Lane M represents the standard protein markers, while lane 1 represents the purified enzyme after application of 10 μL of 2 mg/mL protein sample.

zymes. Through this experiment, productivity of the uricolytic enzyme was tested when some mineral salts ($CaCl_2$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and $MgSO_4 \cdot 7H_2O$) were added to the fermentation medium (Fig. 2h). The maximum enzyme productivity was obtained at 0.9, 0.008, 0.002, 0.002 and 0.005% (w/v) of $MgSO_4 \cdot 7H_2O$ (1,632 mU/mL), $FeSO_4 \cdot 7H_2O$ (1,463 mU/mL), $CaCl_2$ (1,506 mU/mL), $MnSO_4 \cdot 7H_2O$ (1,470 mU/mL) and $ZnSO_4 \cdot 7H_2O$ (1,520 mU/mL), respectively. Data of $MgSO_4 \cdot 7H_2O$ are not shown in Figure 2h due to the difference in the drawing scale. Other researchers also reported that the addition of magnesium sulphate (0.5%) in the fermentation medium enhanced the production of urate oxidase by *Aspergillus terreus*, *Aspergillus flavus*, *Trichoderma* sp. and a mutant *B. subtilis* (Abdel-Fattah & Abo-Hamed 2002; Huang & Wu 2004). In the contrary, Yazdi et al. (2006) reported that tested minerals (Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+}) except Ca^{2+} had no significant effect on enzyme production by LSCs of *M. hiemalis*.

Profile of enzyme purification

As summarized in Table 2 and Figure 3, uricase was purified in several consecutive steps. In the first step,

Table 2. Summary of the uricase purification process.[a]

Purification step	TA (U)	PC (mg)	SA (U/mg)	PF	R (%)
Extracted filtrate	5873	17215	0.34	1.00	100.00
50–80% $(NH_4)_2SO_4$	4213	7832	0.54	1.59	71.73
DEAE-cellulose	3683	3213	1.15	3.38	62.71
Sephadex G-75 FF	2963	1389	2.13	6.26	50.45
Xanthine-agarose	2126	247	8.61	25.32	36.20

[a] TA, total activity; PC, protein content; SA, specific activity, PF, purification folds; R, recovery.

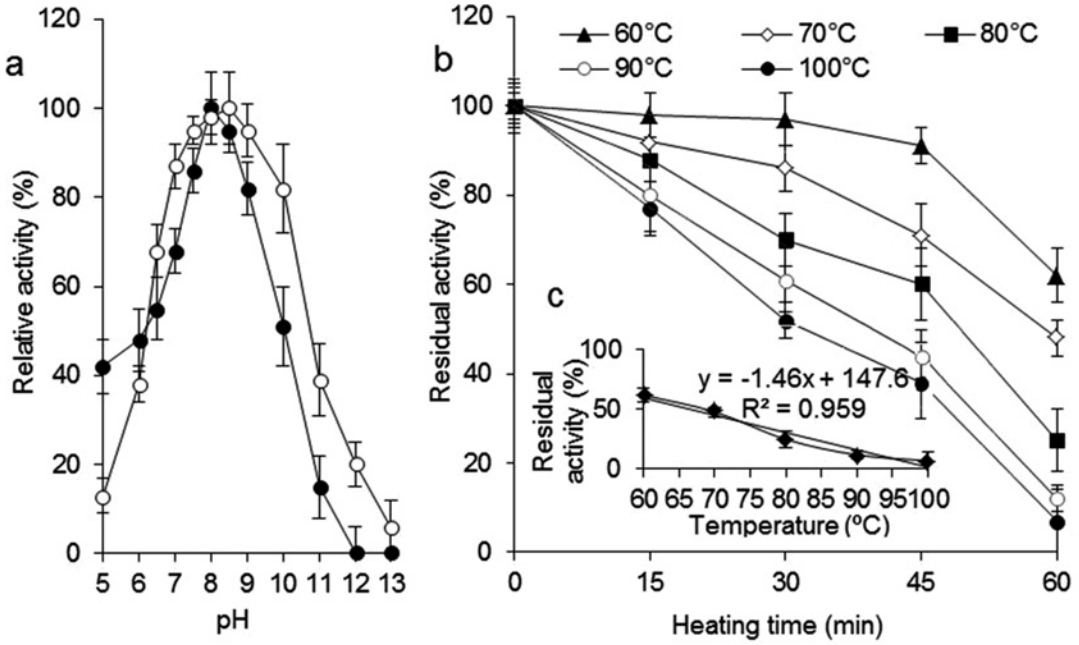

Fig. 4. (a) Profile of pH dependence (full circles) and pH stability (empty circles) of purified uricase. (b) Profile of purified uricase thermal stability at different temperatures. The T_m value was calculated from the plot shown in the panel (c).

enzyme was extracted from the solid contents of flasks and then solid $(NH_4)_2SO_4$ was added. Fractions with $(NH_4)_2SO_4$ concentrations from 50–80% had the highest specific activity. The final specific activity of uricase reached 25.3-fold with 36.2% recovery as compared to the initial extracted filtrate. The concentrated active fractions from the final elution were subjected to SDS-PAGE and showed a prominent band at 33.7 kDa (Fig. 3d), with resemblance to uricases produced by *B. firmus* DWD-33 (33.5 kDa; Kotb 2015a), *C. utilis* (34 kDa; Koyama et al. 1996) and *Microbacterium* sp. strain ZZJ4-1 (34 kDa; Kai et al. 2008), whereas the uricases from *B. fastidious* A.T.C.C. 26904 (36 kDa; Zhao et al. 2006) and *P. aeruginosa* (54 kDa; Ishikawa et al. 2004) were different.

Stability and substrate specificity of the purified enzyme
The pH stability of purified enzyme was tested at range of 5.0–13.0 by incubating enzyme solution for 30 min at 35 ℃ without substrate. At the end of incubation, the remaining activity was measured at pH 8.0 (optimal reaction pH). From the pH stability curve (Fig. 4a), purified uricase appeared catalytically stable between pH 7.0-10.0 coinciding with that reported for uricases of *Microbacterium* sp. ZZJ4-1 (Kai et al. 2008) and *B. firmus* DWD-33 (Kotb 2015a).

The thermal stability of uricase was evaluated by pre-incubation without substrate at 60–100 ℃ for 15–60 min followed by measuring the residual activity at 40 ℃ (optimal reaction temperature) under the standard assay conditions. From thermal stability profile (Fig. 4b,c), tested uricase appeared catalytically stable till 70 ℃ for 30 min and conformational structure seemed not to be affected at 60 ℃ for 15 min as the residual activity was higher than 98% of the original. The higher the temperature was, the higher the denaturation rate of enzyme became as reflected by the values of half-life ($t_{1/2}$) at 60, 70, 80, 90 and 100 ℃ that were 87.0, 56.6, 30.0, 24.2 and 15.6 min, respectively. From thermal inactivation profile (Fig. 4c), the calculated midpoint temperature (T_m) value of uricase was 66.85 ℃. Thermostable uricases have advantages in storage, transportation and *in vitro* clinical applications. In view of uricases that have been used in clinical and diagnostic applications, most of them retained just 50% of their original activity at 60 ℃ and totally denatured above 65 ℃ within 20 min (Schiavon et al. 2000). On the other hand, uricases from *Microbacterium* sp. strain ZZJ4-1 (Kai et al. 2008) and *B. thermocatenulatus* (Lotfy 2008) conserved 100% of their enzymatic ac-

tivity after heat exposure at 70 ℃ for 30 min and 75 ℃ for 45 min, respectively. While, *B. firmus* DWD-33 uricase retained 100% of its initial activity when incubated at 70 ℃ for 60 min (Kotb 2015a).

Another issue concerning the practical use of uricase is its storage stability. An enzyme preparation was stored for 3 months at 4 ℃ in the presence of organic solvents at 20% (v/v) concentration, then the remaining activity was measured at temperature 40 ℃ and pH 8.0. The tested uricase showed the best organic solvent stability with dimethylsulfoxide (112% of original activity) followed by dimethylformamide (101%), acetone (99%), acetonitrile (98%), ethyl acetate (96%), isopropanol (96%), ethanol (95%) and diethyl ether (94%) advocating good activity preservation and biotechnological potential of the current uricase. The stimulation of uricase activity in presence of dimethylsulfoxide could be explained on basis of enhancement of hydrophobic interactions inside polypeptide chains that may consequently induce better conformation for interaction between the active site residues and substrate (Kotb 2015b). The stabilization induced by other organic solvents may be due to replacement of some water molecules inside the core structure of uricase with organic solvent molecules; the enzyme could thus become dehydrated and its quaternary structure conserved. It is often assumed that enzymes with improved solvent stability also become more resistant to other denaturants (Hassanein et al. 2011).

Interestingly, uric acid analogues, such as oxypurinol, allopurinol, xanthine and hypoxanthine that may act as competitive uricase inhibitors due to their structural resemblance with uric acid, did not affect uricase activity. This means that none of the above-mentioned uric acid analogues was a competitive substrate for enzyme, indicating thus the high specificity of the enzyme with respect to uric acid measurement *in vitro* (Kotb 2015a).

Conclusions

The present work indicates that shrimp shell wastes which are cheap and abundant can be used as a substrate for production of value added uricase by *B. subtilis* strain RNZ-79. The final uricase productivity achieved under SSF was 5.05- and 8.0-times higher than the initial productivity under SSF and LSF, respectively. The enzyme may be industrially significant for the *in vitro* clinical applications in measurement of uric acid due to its low value of K_m, pH stability, thermostability, long half-life, storage stability and none of uric acid analogues were found to be the competitive enzyme inhibitor, indicating a high uricase specificity. Future studies will be directed for the *in vitro* application, full characterization, gene expression and studying the relationships between the physiological function and structure of this uricase. Animal study is also in progress for evaluation of uricase ability to dissolve uric acid *in vivo*.

References

Abd El Fattah Y., Saeed H., Gohar Y. & El-Baz M. 2005. Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. Process Biochem. **40:** 1707–1714.

Abdel-Fattah G. & Abo-Hamed N. 2002. Bioconversion of poultry wastes I-factors influencing the assay and productivity of crude uricase by three uricolytic filamentous fungi. New Microbiol. **25:** 57–64.

Adámek V., Králová B., Suchová M., Valentová O. & Demnerová K. 1989. Purification of microbial uricase. J. Chromatogr. **497:** 268–275.

Adámek V., Suchová M., Demnerová K., Králová B., Fořt I. & Morava P. 1990. Fermentation of *Candida utilis* for uricase production. J. Ind. Microbiol. **6:** 85–90.

Alamillo J., Cardenas J. & Pineda M. 1991. Purification and molecular properties of urate oxidase from *Chlamydomonas reinhardtii*. Biochim. Biophys. Acta **1076:** 203–208.

Anderson A. & Vijayakumar S. 2011. Purification and optimization of uricase enzyme produced by *Pseudomonas aeruginosa*. J. Exp. Sci. **2:** 5–8.

Bailey W., Scott E., Finegold S. & Baron E. 1986. Bailey and Scott's Diagnostic Microbiology. Mosby Co., USA.

Baysal Z., Uyar F. & Aytekin C. 2003. Solid state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water. Process Biochem. **38:** 1665–1668.

Bongaerts G., Uitzetter J., Brouns R. & Vogels G. 1978. Uricase of *Bacillus fastidiosus*. Properties and regulation of synthesis. Biochim. Biophys. Acta **527:** 348–358.

Brandenburg J., Wray L., Beier L., Jarmer H., Saxild H. & Fisher S. 2002. Roles of PucR, GlnR, and TnrA in regulating expression of the *Bacillus subtilis* ure P3 promoter. J. Bacteriol. **84:** 6060–6064.

Hassanein W., Kotb E., Awny N. & El-Zawahry Y. 2011. Fibrinolysis and anticoagulant potential of a metallo protease produced by *Bacillus subtilis* K42. J. Biosci. **36:** 773–779.

Hibi T., Hayashi Y., Fukada H., Itoh T., Nago T. & Nishiya Y. 2014. Intersubunit salt bridges with a sulfate anion control subunit dissociation and thermal stabilization of *Bacillus* sp. TB-90 urate oxidase. Biochemistry **53:** 3879–3888.

Huang S. & Wu T. 2004. Modified colorimetric assay for uricase activity and a screen for mutant *Bacillus subtilis* uricase genes following StEP mutagenesis. Eur. J. Biochem. **271:** 517–523.

Ishikawa J., Yamashita A., Mikami Y., Hoshino Y., Kurita H., Hotta K., Shiba T. & Hattori M. 2004. The complete genomic sequence of *Nocardia farcinica* IFM 10152. Proc. Natl. Acad. Sci. USA **101:** 14925–14930.

Kai L., Ma X., Zhou X., Jia X., Li X. & Guo K. 2008. Purification and characterization of a thermostable uricase from *Microbacterium* sp. strain ZZJ4-1. World J. Microbiol. Biotechnol. **24:** 401–406.

Kotb E. 2015a. Characterization of a thermostable uricase isolated from *Bacillus firmus* DWD-33 and its application for uric acid quantification in human serum. Protein Pept. Lett. **22:** 402–409.

Kotb E. 2015b. The biotechnological potential of subtilisin-like fibrinolytic enzyme from a newly isolated *Lactobacillus plantarum* KSK-II in blood destaining and antimicrobials. Biotechnol. Prog. **31:** 316–324.

Koyama Y., Ichikawa T. & Nakano E. 1996. Cloning, sequence analysis, and expression in *Escherichia coli* of the gene encoding the *Candida utilis* urate oxidase (uricase). J. Biochem. **120:** 969–973.

Krishna C. & Chandrasekaran M. 1996. Banana waste as substrate for a-amylase production by *Bacillus subtilis* (CBTK 106) under solid-state fermentation. Appl. Microbiol. Biotechnol. **46:** 106–111.

Laemmli U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.

Liu J., Li G., Liu H. & Zhou X. 1994. Purification and properties of uricase from *Candida* sp. and its application in uric acid analysis in serum. Appl. Biochem. Biotechnol. **47:** 57–63.

Lonsane B., Saucedo-Castaneda G., Raimbault M., Roussos S., Viniegra-Gonzalez G., Ghildyal N., Ramakrishna M. & Krishnaiah M. 1992. Scale-up strategies for solid state fermentation systems. Process Biochem. **27:** 259–273.

Lotfy W. 2008. Production of a thermostable uricase by a novel *Bacillus thermocatenulatus* strain. Bioresour. Technol. **99:** 699–702.

Lowry O., Rosebrough N., Farr A. & Randall R. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:** 265–275.

Montalbini P., Aguilar M. & Ineda M. 1999. Isolation and characterization of uricase from bean leaves and its comparison with uredospore enzyme. Plant Sci. **147:** 139–147.

Nakagawa T., Mazzali M., Kang D., Sanchez-Lozada L., Herrera-Acosta J. & Johnson R. 2006. Uric acid – a uremic toxin? Blood Purif. **24:** 67–70.

Nanda P. & Babu P. 2014. Isolation, screening and production studies of uricase producing bacteria from poultry sources. Prep. Biochem. Biotechnol. **44:** 811–821.

Pandey A., Selvakumar P., Soccol C. & Nigam P. 1999. Solid state fermentation for the production of industrial enzymes. Curr. Sci. **77:** 149–162.

Pandey A., Soccol C. & Larroche C. 2007. Current Developments in Solid-State Fermentation. Springer Science/Asiatech Publishers, New York, USA/New Delhi, India.

Pfrimer P., de Moraes L., Galdino A., Salles L., Reis V., De Marco J., Prates M., Bloch C. & Torres F. 2010. Cloning, purification, and partial characterization of *Bacillus subtilis* urate oxidase expressed in *Escherichia coli*. Biomed. Biotechnol. **2010:** 1–6.

Saeed H., Abdel-Fattah Y., Gohar Y. & Elbaz M. 2004. Purification and characterization of extracellular *Pseudomonas aeruginosa* urate oxidase enzyme. Pol. J. Microb. **53:** 45–52.

Schiavon O., Caliceti P., Ferruti P. & Veronese F. 2000. Therapeutic proteins: a comparison of chemical and biological properties of uricase conjugated to linear or branched poly (ethylene glycol) and poly(N-acryloylmorpholine). Farmaco **55:** 264–269.

Schultz C., Nygaard P. & Saxild H. 2001. Functional analysis of 14 genes that constitute the purine catabolic pathway in *Bacillus subtilis* and evidence for a novel regulon controlled by the PucR transcription activator. J. Bacteriol. **183:** 3293–3302.

Suzuki K., Sakasegawa, S., Misaki H. & Sugiyamac M. 2004. Molecular cloning and expression of uricase gene from *Arthrobacter globiformis* in *Escherichia coli* and characterization of the gene product. J. Biosci. Bioeng. **98:** 153–158.

Swan D., Hale R., Dhillon N. & Leadlay P. 1987. A bacterial calcium-binding protein homologous to calmodulin. Nature **329:** 84–85.

Tanaka A., Yamamura M., Kawamoto S. & Fukui S. 1977. Production of uricase by *Candida tropicalis* using n-alkane as substrate. Appl. Environ. Microbiol. **34:** 342–346.

Wallrath L. & Friedman T. 1991. Species differences in the temporal pattern of *Drosophila* urate oxidase gene expression are attributed to transacting regulatory changes. Proc. Natl. Acad. Sci. USA **88:** 5489–5493.

Werner A. & Witte C. 2011. The biochemistry of nitrogen mobilization: purine ring catabolism. Trends Plant Sci. **16:** 381–387.

Yamamoto K., Kojima Y., Kikuchi T., Shigyo T., Sugihara K., Takashio M. & Emi S. 1996. Nucleotide sequence of the uricase gene from *Bacillus* sp. TB-90. J. Biochem. **119:** 80–84.

Yazdi M., Zarrini G., Mohit E., Faramarzi M., Setayesh N., Sedighi N. & Mohseni F. 2006. *Mucor hiemalis*: a new source for uricase production. World J. Microbiol. Biotechnol. **22:** 325–330.

Yokoyama S., Ogawa A. & Obayashi A. 1988. Rapid extraction of uricase from *Candida utilis* cells by use of reducing agent plus surfactant. Enzyme Microb. Technol. **10:** 52–55.

Zhao Y., Zhao L., Yang G., Tao J., Bu Y. & Liao F. 2006. Characterization of a uricase from *Bacillus fastidious* A.T.C.C. 26904 and its application to serum uric acid assay by a patented kinetic uricase method. Biotechnol. Appl. Biochem. **45:** 75–78.

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