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Isolation, partial purification and characterization of thermophilic uricase from *Pseudomonas otitidis* strain SN4

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ABSTRACT

Aims: The present study aimed at isolating new source of uricase producers from Malaysian hot springs together with partial purification and characterization of thermophilic uricase from novel strain.
Methodology and results: A bacteria strain, designated as SN4, was found to have the ability to degrade uric acid. 16S rRNA analysis identified SN4 as *Pseudomonas otitidis*. Uricase was then extracted from SN4 and purification was performed via ammonium sulphate precipitation. The effects of temperature, pH and metal ions on partially purified uricase were evaluated. Results showed that 70% ammonium sulphate concentration gave the highest uricase activity at 4.18 U/mL compared to other concentrations. The molecular weight of the partially purified uricase was 33 kilodalton (kDa). The optimum temperature for uricase was 45 °C and its activity was highest at pH 8.0. Calcium ions and copper ions enhanced uricase activity while cobalt ions reduced uricase activity.
Conclusion, significance and impact of study: Isolation and investigation of uricase producers from new sources such as thermophiles would increase availability and thermal stability of the uricase that could be used for significant purposes such as in biochemical and clinical applications.

Keywords: Uricase, uric acid, *Pseudomonas otitidis*, hot springs

INTRODUCTION

Uricase or formerly known as urate oxidase (E.C. 1.7.3.3) oxidizes uric acid into a more soluble form which is allantoin. This enzyme is mainly found in mammals (Keilin, 1959), plants (Montalbini *et al.*, 1997), fungi, (Montalbini *et al*., 1999), yeasts (Adamek *et al*., 1990) and bacteria (Yamamoto *et al.*, 1996). Uricase has been used as a diagnostic reagent in clinical and biochemical fields for measurement of uric acid in blood and other biological fluids.

Uric acid is a final product of purine catabolism in human body and excreted out by the kidneys. However, lack of functional uricase and excretion of uric acid lead to various health problems such as gout, hyperuricemia, cardiovascular risk as well as renal failure (Anderson and Vijayakumar, 2011).

Uricase has been isolated from various types of bacteria such as *Escherichia coli* (Nakagawa *et al*., 1996), *Brevibacterium* (Kida and Kunihisa, 1966), *Micrococcus*, *Bacillus* sp. including *Bacillus fastidiosus* (Bongaerts *et al*., 1978), *B. pasteurii* (Christians and Kaltwasser, 1986) and *B. subtilis* (Huang and Wu, 2004) and *Pseudomonas* sp. (Ishikawa *et al*., 2004). A recombinant uricase from *A. flavus* expressed in *Saccharomyces cerevisiae* known as Rasburicase has been found able to act rapidly and safely in the treatment of gout and hyperuricemia (Bomalaski and Clark, 2004).

Yazdi *et al. (*2006) claimed that although uricase produced from many sources, due to its increasing importance for treatment and diagnosis, it is still necessary to screen new sources of uricase producers. Isolation and identification of microorganisms from different sources could contribute to the discovery of potential uricase suited for other biochemical diagnosis and clinical analysis.

Temperature has a great effect on enzyme activity and it is important to withstand with high temperature. Thus, thermostability enzymes will have advantages in transportation and storage. In addition, thermostability enzyme is crucial in clinical applications. Suzuki *et al.* (2004) notes that although many uricases have been isolated from microorganisms, their thermostabilities have been reported to be low. The use of thermostable uricase is desirable particularly for diagnostic purposes.

The objectives of this study were to isolate new sources of uricase producers from Malaysian hot springs. Considering that this enzyme has potential value in applications, the present study was undertaken to partially purify uricase. The optimum conditions for uricase from

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SN4 were also determined by function of temperature, pH and metal ions. To our knowledge this is the first report on the isolation of thermophilic uricase from *P. otitidis* isolated from hot springs.

MATERIALS AND METHODS

Sampling

Hulu Langat, Selangor, Malaysia was chosen as the hot springs site for microorganism isolation. The temperature of the sampling site is approximately 74 °C. Water and biomat samples were kept in sterile bottles and kept in dry ice and stored in cold room until used.

Isolation and screening of bacterial strain

Water and biomat samples were aseptically inoculated into sterile (0.8% w/v) uric acid broth, and then incubated at 37 °C in a shaking incubator. The uric acid medium contained 0.8% (w/v) uric acid, 0.01% (w/v) NaCl, 0.2% (w/v) K_2HPO_4, 0.05% (w/v) KH_2PO_4, and 0.01% (w/v) $CaCl_2$. The pH was adjusted to 7.5. The culture was then streaked onto Luria-Bertani agar (LBA) plate and incubated at 37 °C for 24 h. Repeated streaking of bacterial colonies was performed onto new agar plate till pure colonies were isolated. All the isolates were further screened for their ability to degrade uric acid on uric acid agar plate. Screening was conducted by pipetting 3 µL of overnight culture onto uric acid plates. The plates were incubated at 37 °C for 24 h. The clearing zone was measured after 24 h of incubation indicating uric acid degradation.

Identification of bacterial strain

A DNA extraction kit from Promega was used for genomic DNA extraction. The 16s rRNA gene of the uric acid degrading bacteria was amplified using the forward primer 5'- AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-AAGGAGGTGATCCAGCCGCA-3'. The amplification process was conducted using the following PCR programmes: initial denaturation for 4 min at 95 °C, denaturation for 30 sec at 95 °C, annealing for 60 sec at 55 °C, extension for 4 min at 72 °C and final extension for 10 min at 72 °C. This cycle was repeated until 30 times and held at 4 °C. The purified PCR product was sent to First Base Laboratories Sdn. Bhd. (Malaysia) for sequencing. The sequence obtained from sequencing was analyzed using the Basic Local Alignment Search Tool (BLAST) to identify the species. Phylogenetic trees of SN4 were then constructed and established using the Ribosomal Database Project (RDP) to determine the phylogenetic relation.

Preparation of crude intracellular uricase

SN4 was grown overnight at 37 °C, 150 rpm. Cells were harvested by washed twice in an equal volume of 0.9% (w/v) saline solution and centrifuged at 8 000 rpm for 15 min at 4 °C. The cells were then resuspended in 0.1 M borate buffer (pH 8.5) and disrupted by an ultrasonic device at 5% amplitude at 4 °C to lease the enzyme. Disrupted cells were then centrifuged again at same condition.

Partial purification of enzyme

The inoculum was grown in four litres of uric acid medium until late exponential phase. The culture was then harvested via centrifugation and washed twice with 50mM phosphate buffer (pH 7.5). The cells were then resuspended with 0.1 M phosphate buffer (pH 7.0) at 4 °C and disrupted by ultrasonic device at 5% amplitude at 4 °C. Partial purification of uricase enzyme was then achieved by ammonium sulphate precipitation followed by dialysis. The cell free extract was saturated with solid ammonium sulphate up to 80%. The precipitate from 30%-80% fractions saturation was then centrifuged (40 min, 8 000 rpm at 4 °C) and pellet was collected for further analysis. The enzyme mixture was dissolved in a buffer of 2:1 (volume of the buffer:weight of the precipitate) ratio and transferred to a dialysis bag. The content was immersed in sodium phosphate buffer and continuously stirred using a magnetic stirrer for 24 h. The phosphate buffer was changed three times during the process in order to obtain proper purification. The purified enzyme was then collected for further analysis (Anderson and Vijayakumar, 2011).

Molecular mass determination

The relative molecular mass of the uricase was determined by SDS-PAGE with 10% polyacrylamide gels (Laemmli, 1970). The sizes of the protein were estimated by comparing them with marker proteins of known molecular weight. Samples of crude enzyme, 30%, 40% and 70% ammonium sulphate precipitated enzyme solution were loaded in the well and ran at constant voltage of 150 V for 45 min. The protein was then stained with Pierce Imperial™ Protein Stain for 1 h and destained in distilled water for overnight.

Enzyme assay for uricase enzyme

Uricase assay was evaluated spectrophotometrically by complying urate-peroxidase method (Masaru, 1981). The assay was measured based on the oxidation of uric acid into allantoin and H_2O_2. Crude enzyme (0.1 mL) was incubated in 0.6 mL sodium borate buffer (pH 8.5, 0.1 M) containing 2 mM uric acid, 0.15 mL 4-aminoantipyrine (30 mM), 0.1 mL phenol (1.5%) and 0.05 mL peroxidase (15 U/mL). The mixture was incubated at 37 °C for 20 min. In order to stop the reaction, 1 mL of ethanol was added. An absorbance of 540 nm was read using a spectrophotometer. One unit of uricase was equivalent to the amount of enzyme that produces 1.0 µL of H_2O_2 per minute.

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Characterization of enzyme

Effect of pH and temperature

The effect of pH was studied to determine the optimal pH for uricase production. The pHs were adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. Uricase productions at different temperatures were also studied. The enzyme reaction was pre-incubated at temperatures between 20-70 °C at 5 °C intervals, and the enzyme solution was then added and incubated for 20 min at the same temperature to measure its activity.

Effect of metal ions

Metal ions Co^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} were chosen to study the effect of metal ions in the uricase production. Metal ions were applied at 1 mM final concentration. The enzyme reaction was mixed with different metal ions solution and incubated at 37 °C for 20 min. The enzyme mixture without metal ions was act as a control. After the incubation period, uricase activity was determined and compared with the control.

RESULTS

Isolation and screening of uricase degrading microbes

Water samples from the hot springs were aseptically inoculated into uric acid broth and incubated at 37 °C. The water samples which showed positive growth in uric acid broth were subsequently streaked onto LBA plates. Four different colonies labelled as SN1, SN2, SN3 and SN4 showed significant growth in the presence of uric acid. All isolates were further screened based on the width of clear zone on uric acid agar plates. Figure 1 shows the clearing zones formed on uric acid agar plate after 24 h incubation. The production of clearing zones indicated that bacterium SN4 was capable of degrading uric acid.

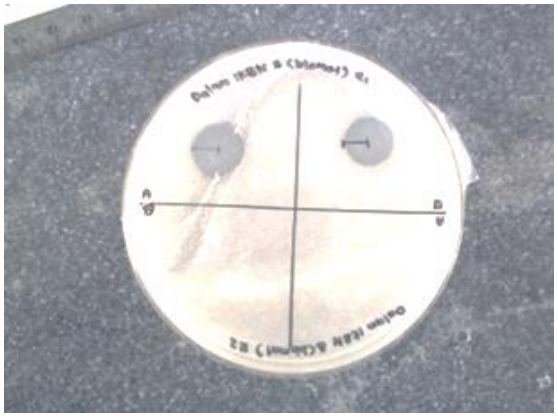

Figure 1: The clearing zone formed on uric acid agar plate after 24 h incubation. This showed uric acid degradation by bacterium SN4.

Identification of microbes

The DNA of SN4 was extracted and the 16S rRNA sequences were then analysed. Analysis results showed 99% similarity of SN4 with *P. otitidis* sequences from NCBI (National Centre Biotechnology Information) databases. The SN4 nucleotide sequence was submitted to GenBank and assigned the following accession number KM196557. In order to determine the phylogenetic relation of the isolates with *P. otitidis,* a phylogenetic tree was constructed and established using Ribosomal Database Project (RDP). Figure 2 shows the relationship between SN4 and *Pseudomonas* sp.

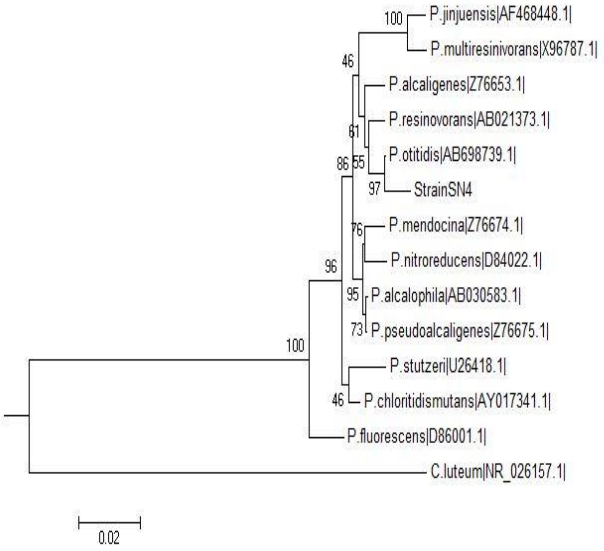

Figure 2: Phylogenetic position of strain SN4 based on 16s rRNA sequence. Numbers at each point of the branch are the bootstrap values.

Partial purification of enzyme

Uricase produced from SN4 was purified using different concentrations of ammonium sulphate (20%-80%). From the results, 70% ammonium sulphate precipitation showed the highest uricase activity at 4.18 U/mL while no enzyme activity was detected in 20%, 50% and 60% ammonium sulphate precipitations. This finding correlated with previous studies where 70% ammonium sulphate concentration showed the highest activity compared to other fractions (Atalla *et al*., 2010; Anderson and Vijayakumar, 2011).

Molecular mass determination

Uricase size from crude extract, 30%, 40% and 70% of ammonium sulphate precipitation was then estimated by SDS-PAGE. A standard protein marker of known molecular weight was used. Figure 3 shows that the

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estimated uricase molecular weight was approximately 33 kDa. This finding correlates with previous study which claimed that the estimated molecular weight of uricase was between 33-35 kDa (Kai *et al*., 2007).

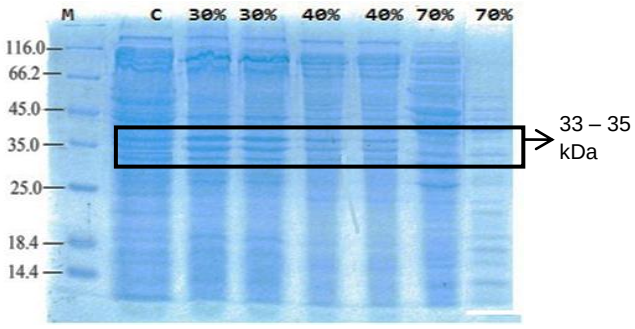

Figure 3: Lane 1, Protein Marker; Lane 2, Crude extract, Lanes 3 and 4, 30% precipitation; Lanes 5 and 6: 40% precipitation; Lane 7 and 8, 70% precipitation.

Uricase assay

Hydrogen peroxide was formed by the action of uricase on uric acid reacts with 4-aminoantipyrine and phenol in the presence of peroxidase. The enzyme reaction produced red colored product known as quinoneimine dye. The formation of quinoneimine dye was proportional to the amounts of uric acid degraded by uricase (Machida and Nakanishi, 1980).

Effect of pH and temperature

The purpose of these experiments was to study the trend and determine the optimum pH and temperature of our enzyme. The experiments relating to pH and temperature were conducted independently of each other as shown in Figures 4 and 5. Thus, statistical analysis to indicate significant differences between these two parameters is irrelevant. Figure 4 illustrated that uricase exhibits the highest activity at 45 °C while Figure 5 showed optimum pH for uricase activity of SN4 was pH 8.0 and the activities gradually decreased when the pH increased.

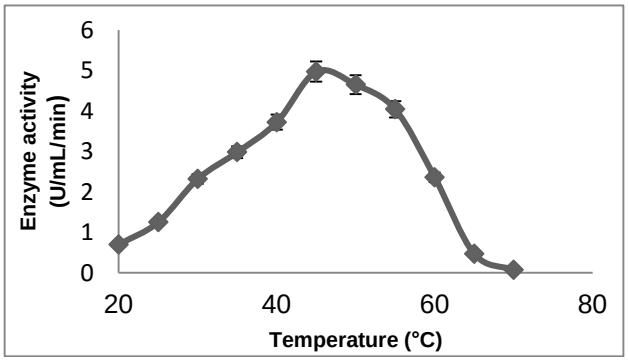

Figure 4: Effect of different temperature on uricase activity. Crude enzyme solution was incubated in water bath at different temperatures for 30 min respectively. The remaining activities were measured after cooling to 37 °C. The values are mean of triplicates with standard error bars.

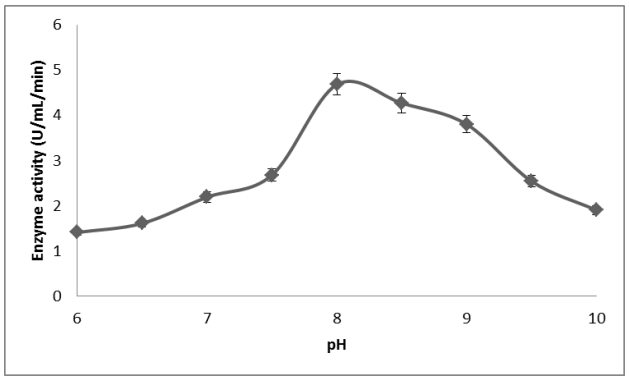

Figure 5: Effect of different pH on the activity of uricase produced by SN4. The values are mean of triplicates with standard error bars.

Effect of metal ions

The purpose of this experiment was also to study the trend and determine the effect of metal ions on enzyme activity. Thus, statistical analysis to indicate significant differences between parameters is irrelevant. Figure 6 shows the influence of different metal ions was also studied and results indicated that calcium ions increased the enzyme activity to 140%. In contrast, manganese, magnesium, ferrous and copper ions did not give significant effect to the activity.

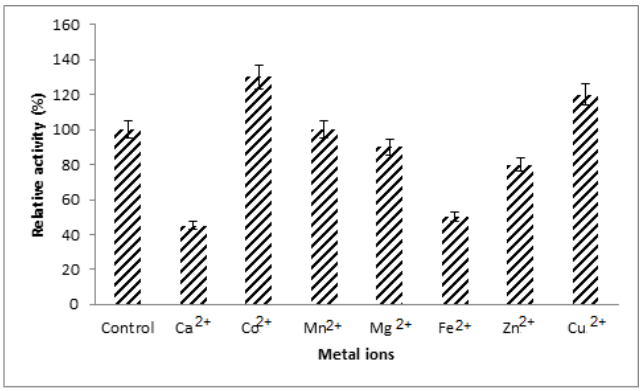

Figure 6: Effect of different metal ions on the activity of uricase produced by SN4. Data shown is the mean values of triplicates with standard error bars.

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DISCUSSION

In the present study, SN4 was isolated from hot springs and grown in a medium containing uric acid. A positive microbial growth turned insoluble uric acid broth into a colourless soluble solution. From the screening study tested on uric acid agar plate, formation of clearing zone on the uric acid agar plate was observed after 24 h incubation. From the observation, clear hydrolysis zone was formed around the culture. The appearance of clearing zone indicates that uric acid was hydrolyzed into a more soluble form, and uricase was produced by the isolates. SN4, which formed the largest clearing zone approximately 7 mm in diameter within 24 h incubation was selected and used for further studies. SN4 showed 99% similarity with *P. otitidis* sequences from NCBI (National Centre Biotechnology Information).

Pseudomonas otitidis was firstly proposed and isolated by Clark *et al.* (2006) from clinical specimens of infected human ears. This strain is a new *Pseudomonas* species that has been recognized in otic infections in human. *P. otitidis* was also found to be genotypically and phenotypically closely related to *Pseudomonas aeruginosa*. *Pseudomonas otitidis* becomes new species that has ability to produce uricase. To the best of our knowledge, this study is the first report on the production of thermophilic uricase from *P. otitidis* which was isolated from hot springs as many previous studies reported uricase were isolated from poultry wastes.

Ammonium sulphate precipitation was performed for the protein solution at different concentrations which were 20% (w/v), 30% (w/v), 40% (w/v), 50% (w/v), 60% (w/v), 70% (w/v) and 80% (w/v) ammonium sulphate saturation. This was done in order to recognize a suitable concentration of ammonium sulphate in which the protein would aggregate and precipitate out. Dialysis was then performed in order to remove ammonium sulphate from the protein solution as ammonium sulphate can interact with proteins and cause denaturation.

The results given in Figure 3 show that the molecular weight of uricase was estimated to be 33 kDa. This finding is in agreement with previous studies (Kai *et al*., 2007; Anderson and Vijayakumar, 2011) whereby the uricase size was estimated to be in the range of 33-35 kDa. In contrast, a study from Hesham *et al*. (2004) found a larger size of purified uricase from *Pseudomonas aeruginosa* which was 68.0 kDa for one subunit while uricase from *Bacillus fastidiosus* was reported to be 145-150 kDa (Bongaerts and Vogel, 1976).

Most enzymes work optimally at 37 °C. Interestingly, in this study, uricase activity was found to be optimum at 45 °C (4.97 U/mL.min). The enzyme activity was then decreased at 70 °C (0.07 U/mL.min). This was likely due to the high temperature that caused protein denaturation or instability. A study on *Gliomastix gueg* reported 35 °C as the optimum temperature for uricase (Mabrouk *et al*., 2010) while study *on Microbacterium* sp. strain ZZJ4-1 reported an optimum temperature at 30 °C. The characteristics of thermophilic organisms refer to the capability in producing thermostable enzyme. Thermophilic enzymes are needed as they can maintain their activity and reduce risk of microbial contamination. Thus, thermophilic enzymes have great interests in commercial and industrial applications (Kuchner and Arnold, 1997).

pH plays an important role in enzyme function and activity. The point value where the enzyme is most active is known as the optimum pH. The optimum pH for SN4 was tested and results showed that pH 8 was the optimum pH where uricase activity reached its highest activity (4.68 U/mL.min). The literatures have reviewed varied optimal pH for uricase depending on the species producing the enzyme. For example Kai *et al.* (2007) reported an optimum pH of 9 for *Microbacterium* sp. strain ZZJ4-1.

Metal ions play important roles in the biological function of many enzymes. Metal can either act as an electron donor or acceptor. Metal ions can catalyse or inhibit enzyme reaction (Coolbear *et al*., 1992). In order to study the effect of metal ions on uricase activity, different metal ions were chosen (Co^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}). From this study, it was found that Ca^{2+} and Cu^{2+} enhanced the enzyme activity slightly while other metal ions seemed to inhibit the activity. Previous studies reported that Ca^{2+} enhanced uricase activity (Atalla *et al*., 2010) whilst another study showed that Ca^{2+} and Cu^{2+} ions enhanced the activity of uricase (Hesham *et al.*, 2004). Atalla *et al.* (2010) suggested that metal ions bind to the uricase and changes the enzyme's activity by stabilization or destabilization the enzyme's conformation.

CONCLUSION

This study has successfully isolated *P. otitidis* SN4 from Malaysian hot springs able to produce uricase. Interestingly, this is the first report on the production of uricase from *P. otitidis*. Uricase is one of the most important enzymes used in the detection of uric acid in blood and other biological fluids. Thermophilic uricase of *P. otitidis* SN4 has commercial potential to be used in biosensor field particularly for uric acid detection.

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