Thermostable Xanthine Oxidase Activity from *Bacillus pumilus* RL-2d Isolated from Manikaran Thermal Spring: Production and Characterization

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Abstract Xanthine oxidase is an important enzyme of purine metabolism that catalyzes the hydroxylation of hypoxanthine to xanthine and then xanthine to uric acid. A thermostable xanthine oxidase is being reported from a thermophilic organism RL-2d isolated from the Manikaran (Kullu) hot spring of Himachal Pradesh (India). Based on the morphology, physiological tests, and 16S rDNA gene sequence, RL-2d was identified as *Bacillus pumilus*. Optimization of physiochemical parameters resulted into 4.1-fold increase in the xanthine oxidase activity from 0.051 U/mg dcw (dry cell weight) to 0.209 U/mg dcw. The xanthine oxidase of *B. pumilus* RL-2d has exhibited very good thermostability and its $t_{1/2}$ at 70 and 80 °C were 5 and 1 h, respectively. Activity of this enzyme was strongly inhibited by Hg^{2+} , Ag^{+} and allopurinol. The investigation showed that *B. pumilus* RL-2d exhibited highest xanthine oxidase activity and remarkable thermostability among the other xanthine oxidases reported so far.

Keywords *Bacillus pumilus* · Xanthine oxidase · Thermophilic microorganisms · Thermostability

Introduction

Xanthine oxidase is a complex molybdo-flavo enzyme which catalyzes the oxidation of hypoxanthine to xanthine and then xanthine to uric acid, and plays a vital role in the purine nucleotide metabolism [1, 2]. It was first identified

in milk and forms a major component of the milk fat globule membrane [2]. This enzyme has been reported from several bacterial genera such as *Streptomyces*, *Pseudomonas*, *Comamonas*, *Eubacterium*, *Clostridium*, *Micrococcus* [2] and fungal genera such as *Rhizoctonia*, *Sclerotinia*, *Sclerotium* [3] as well as from some plants, metazoans and animals [4, 5]. There are only few reports on thermostable xanthine oxidase from microbial sources [6].

Xanthine oxidase from various sources are proteins of molecular weight ~300 kDa and mainly contain molybdopterin cofactors [2, 8]. Each enzymatic unit contains flavin adenine dinucleotide, molybdenum and iron sulfur centres [2]. Electron transfer reactions are mainly due to ferredoxin iron-sulfur [2Fe–2S] cluster [7]. The catalytic reaction of xanthine oxidase is initiated by abstraction of a proton from the Mo–OH group by a conserved active site glutamate residue. The oxidative hydroxylation of xanthine to uric acid takes place at the molybdenum centre and results in the two-electron reduction of the metal from Mo(VI) to Mo(IV). The enzyme is subsequently re-oxidized by NAD^{+} or molecular oxygen in a reaction that occurs at the FAD [7].

Xanthine oxidase is used in the oxidation of purines and related compounds and plays a role in biochemical reactions such as hydroxylation of purines, pterines, aromatic heterocycles, aliphatic and aromatic aldehydes and also in the detoxification or activation of endogenous compounds and xenobiotics [4]. The purified enzyme is used in the assay method of super oxide dismutase [8]. Xanthine oxidase also has role in the food industries for assessment of freshness of meat [9]. The inhibitor of xanthine oxidase i.e. allopurinol is used as a drug for inhibiting xanthine oxidase in gout disease [10, 11].

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In the present communication, isolation of a novel source of thermostable xanthine oxidase—*B. pumilus* RL-2d from Manikaran thermal spring of Himachal Pradesh, India, optimization of xanthine oxidase production and its characterization are being reported.

Materials and Methods

Chemicals

All the chemicals were of analytical grade. The xanthine and media components were purchased from Hi Media (India) and the inorganic salts were from Merck (India).

Sample Collection

The soil samples for the isolation of thermophilic bacteria for xanthine oxidase activity were collected aseptically from Manikaran hot spring (80–95 °C) in sterile screw capped tubes and stored at 4 °C for further processing. The samples were processed within 24 h of collection.

Isolation of Bacteria for Xanthine Oxidase Activity

Enrichment culture technique method was used for the isolation of thermostable xanthine oxidase producing bacteria. One gram soil samples were added into a 50 ml of mineral salt medium (MSM) supplemented with 1 mM/l xanthine as sole source of carbon and nitrogen. The MSM broth contained (g/l) $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 2.5; KH_2PO_4 , 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.06; and yeast extract, 0.01, pH 7.5. The culture was allowed to grow for 3 days at 55 °C in shake flask at 150 rpm. Subsequently, the culture broth after third enrichment was serially diluted to 10^{-4} – 10^{-6} times with physiological saline and plated on MSM plates having agar (3 %) and xanthine (1 mM/l) and were incubated at 55 °C for 48 h. Positive isolates were selected on the basis of growth on the selective plates and organisms obtained in this way were maintained on nutrient agar plates (0.5 % peptone, 0.3 % yeast extract, 0.1 % beef extract, 0.5 % sodium chloride, 0.05 % xanthine and agar 3 %, pH 7.5) and stored at 4 °C.

Screening of Bacterial Isolates for Xanthine Oxidase Activity

The culture was grown aerobically in 50 ml of nutrient broth medium supplemented with xanthine and incubated at 55 °C and 150 rpm for 24 h. Two percent (v/v) preculture was added into 50 ml of production medium

containing nutrient broth supplemented with xanthine and were incubated under the same conditions for 24 h and then harvested by centrifugation at $12,000 \times g$ for 5 min at 4 °C. The cells were washed thrice with 0.05 M Tris–HCl buffer (pH 7.5) and suspended in the same buffer and these resting cells were used for xanthine oxidase activity.

The hyper-active bacterial isolate RL-2d was identified by physiological and biochemical tests and finally by 16 S rDNA gene sequencing.

Characterization and Strain Identification

The physiological and biochemical tests were carried at Institute of Microbial Technology (IMTECH), Chandigarh, India. The microorganism was tentatively identified by gram staining and other morphological and biochemical tests such as spore formation, acid production (from dextrose, fructose, mannitol, trehalose, xylose, inulin, maltose, sorbitol and mannose), hydrolysis by casein and starch. Catalase, indole and methyl red tests were also done for the characterization of microorganism. Finally, the strain was identified by 16S rDNA gene sequence analysis. Genomic DNA was extracted from bacterial culture and the 16S rDNA gene was amplified by PCR using universal primers 27 F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1541 R (5'-AAG GAG GTG ATC CAG CCG CA-3'). The PCR products were purified and sequenced by Xcelris Labs Limited, Ahmedabad, India. Related sequences were obtained from the GenBank database (National Centre for Biotechnology Information [NCBI], USA) using the BLAST search program. The sequences were aligned using multiple sequence alignment software, CLUSTAL W ver. 1.81. A phylogenetic tree was constructed using MegAlign software (DNASTAR, Inc., Madison, WI, USA).

Assay of Xanthine Oxidase Activity

Xanthine oxidase activity in resting cells of *B. pumilus* RL-2d was assayed using the method developed by Agarwal and Banerjee [4]. Test reaction contained 500 μl (2.7×10^5 microbial cells) cell suspension, lysed with NaOH (1 mol/l); 500 μl nitroblue tetrazolium (NBT) solution containing 50 mmol/l Tris–HCl, pH 7.6, 1 mmol/l xanthine and 0.50 mmol/l NBT while in the control experiment, 500 μl of cell suspension was incubated with allopurinol (50 mmol/l) for 5 min at 55 °C and to this mixture, 500 μl NBT solution was added and both the reaction mixture were incubated for 5 min at 55 °C, centrifuged and absorbance of supernatant was measured by spectrophotometer at 540 nm. Positive control was set up with commercially available xanthine oxidase from bovine milk, keeping all the reaction parameters same.

Optimization of Culture Parameters for Production of Xanthine Oxidase

Following culture parameters were optimized using one variable at a time (OVAT) approach.

Selection of Medium

Bacillus pumilus RL-2d was cultured in seven (M1–M7) different media, out of which four were already reported in literature and rest were formulated in the present investigation. Fifty milliliter of nutrient broth (pH 7.5) was used as a seed medium and culture was incubated at 50 °C for 24 h in an incubator shaker (160 rpm). Subsequently, two percent of inoculum was transferred into 250 ml Erlenmeyer flasks containing 50 ml of M1–M7 as production media, respectively. These flasks were incubated at 50 °C for 24 h at 160 rpm in an incubator shaker. After 24 h, cells were harvested by centrifugation (10,000×g for 10 min at 4 °C), washed with Tris–HCl buffer (0.05 M, pH 7.5) and suspended in the same buffer and xanthine oxidase activity was assayed.

Effect of Carbon Sources

To study the effect of different carbon sources on the production of xanthine oxidase, the growth media (M7, pH 7.5) was supplemented with 1 % (w/v) of different carbon sources (glucose, sucrose, glycerol, maltose, galactose, sodium succinate, sodium acetate, fructose and lactose). The inoculum was prepared by transferring a loop full of *B. pumilus* RL-2d culture from slant to 50 ml of nutrient broth (pH 7.5) in 250 ml Erlenmayer flask and incubated at 50 °C at 150 rpm. Two percent of inoculum was added to 50 ml of growth medium (supplemented with different carbon sources) and incubated at 50 °C for 24 h in an incubator shaker at 160 rpm. Xanthine oxidase activity in the resting cells was assayed as described in the preceding section.

Effect of Nitrogen Source and Concentration

To evaluate the effect of nitrogen sources on the production of xanthine oxidase, various organic and inorganic nitrogen sources (peptone, beef extract, tryptone, yeast extract, urea, ammonium nitrate, ammonium chloride and ammonium sulphate) were added in the medium. After selection of suitable nitrogen source, this was used at different concentrations (1.0–4.0 %) in the production medium to optimize its concentration.

Effect of Medium pH

The organism was grown in a pH range of 5.0–11.0 to investigate the effect of pH on the production of xanthine

oxidase. Cells were harvested after 24 h and enzyme activity was assayed as described in the preceding section.

Effect of Production Temperature

B. pumilus RL-2d was grown at different temperatures ranging from 40 to 70 °C. Two milliliter of 24 h grown preculture was inoculated to 50 ml of selected production medium and flasks were incubated at 40–70 °C for 24 h. Cells were harvested after 24 h and enzyme activity was assayed.

Age of Inoculum

The production medium was inoculated with the inoculum of the different ages (12–40 h) and incubated at 55 °C to determine the optimum age of inoculum for the production of xanthine oxidase. Enzyme activity was assayed as described earlier.

Incubation Time

In order to find out the appropriate duration of incubation for the production of xanthine oxidase activity by *B. pumilus* RL-2d, the organism was cultured under hitherto optimized culture conditions for 40 h and the culture samples were withdrawn at an interval of 4 h till 40 h and the xanthine oxidase activity was assayed.

Optimization of Reaction Conditions for the Assay of Xanthine Oxidase Activity

Buffer System and pH

In order to find out the optimum pH, the reaction was performed in various buffers of different pH at 55 °C for 5 min. The various buffers [Citrate buffer (pH 4.0–6.0), Sodium phosphate (pH 6.0–8.0), Potassium phosphate (pH 6.0–8.0), Borate buffer (pH 7.5–9.0), Tris–HCl buffer (pH 7.0–8.5) and Carbonate buffer (pH 9.0–10.0)] were used at a concentration of 0.05 M.

Reaction Temperature

The xanthine oxidase activity was assayed in 0.05 M Tris–HCl buffer (pH 7.5) at different reaction temperatures (50–80 °C).

Thermal Stability

Thermal stability of crude enzyme of *B. pumilus* RL-2d was evaluated by incubating the enzyme at different temperatures (40, 50, 60, 70, 80 and 90 °C) for 6 h, samples

were withdrawn after an interval of 1 h and xanthine oxidase activity was assayed.

Effect of Metal Ions and Inhibitors

The effect of different metal ions (FeCl_3 , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, CoCl_2 , $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, AgNO_3 , HgCl_2 , $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) and inhibitor (allopurinol) on enzyme activity were tested by pre-incubating the enzyme at 1 mM concentration of metal ions/inhibitor for 30 min at room temperature.

Substrate Concentration

The effect of substrate (xanthine) concentration ranging from 0.1 to 1.0 mM on the activity of xanthine oxidase of *B. pumilus* RL-2d was studied.

Results

Morphological and Biochemical Characteristics

Bacillus pumilus RL-2d was isolated from soil sample from Manikaran hot spring in our laboratory as thermophilic xanthine degrading bacterium. It has been found that the *B. pumilus* RL-2d is a Gram positive, long rod shaped, spore forming, motile, thermophilic (40–65 °C) and grows mostly at basic pH (6.8–11) with maximum growth at pH 7.5. The isolate is aerobic in nature, produced acid from dextrose, fructose, mannitol and trehalose but do not ferment xylose, inulin, maltose, sorbitol and mannose. The bacteria hydrolyze casein but not starch. The isolate did not show growth on MacConkey agar and showed negative test for indole and methyl red but positive result for catalase.

16S rDNA Gene Sequencing

The sequence obtained by 16S rDNA gene sequencing was deposited in the GenBank database with accession No. KF752495. Based on the 16S rDNA phylogenetic analysis, the isolate RL-2d was related (98 % sequence identity) mostly to *Bacillus pumilus* strain HT-Z 41 (KJ526890.1) (Fig. 1). By examining physiological and biochemical characteristics and comparing its 16S rDNA gene sequence, the strain was identified as *Bacillus pumilus* and named *Bacillus pumilus* RL-2d.

Optimization of Culture Parameters for Production of Xanthine Oxidase by *B. pumilus* RL-2d

Various culture parameters like medium composition, carbon source, nitrogen source, temperature, pH, etc. were optimized to enhance the production of xanthine oxidase of *B. pumilus* RL-2d.

Medium

B. pumilus RL-2d was cultured in 7 different media (Table 1) and medium M6 (pH 7.5) containing (g/l) 10.0 g glucose, 3.0 g yeast extract, 1.0 g beef extract, 5.0 g peptone, 5.0 g sodium chloride and 0.152 g xanthine proved to be the best for the xanthine oxidase activity (0.051 U/mg dcw).

Carbon Source

The maximum xanthine oxidase activity (0.056 U/mg dcw) was recorded in medium containing glycerol as a carbon source (Fig. 2a). This means that extra carbon source increases the xanthine oxidase activity and also the cell biomass.

Fig. 1 Phylogenetic dendrogram based on the 16S rDNA gene sequences. Numbers in parentheses are accession numbers of published sequences. KF 752495.1: *Bacillus pumilus* RL-2d, KJ 526890.1: *Bacillus pumilus* Strain HT-Z41, KF 052985.1: *Bacillus*

stratosphericus Strain, KC 915229.1: *Bacillus pumilus* Strain ESR 21, EU 520307.1: *Bacillus* sp. 01105, AF 001963.1: *Anoxybacillus ayderensis*, NR_026516.1: *Anoxybacillus flavithermus* strain DSM 2641, AB 379690.1: *Pseudomonas* sp. EC-V30-5

Table 1 Screening of various media for the production of xanthine oxidase activity by *B. pumilus* RL-2d

Medium no.	Enzyme activity (U/mg dcw)	Dry cell mass (mg dcw/ml)	References
M1	0.040	3.5	[14]
M2	0.001	2.0	Mineral salt media
M3	0.013	3.0	[6]
M4	0.011	2.0	[15]
M5	0.038	3.5	Nutrient broth
M6	0.051	2.5	Formulated in the study
M7	0.009	2.0	[2]

Nitrogen Source

Various nitrogen sources were added to the medium and maximum xanthine oxidase activity (0.086 U/mg dcw) was observed in medium containing yeast extract (Fig. 2b). The enzyme production from *B. pumilus* RL-2d and the cell biomass was low in presence of inorganic nitrogen sources. The yeast extract was further used at different concentrations (1.0–4.0 %) in the production medium and optimum concentration was found to be 3.0 % (Fig. 2c).

Optimization of Production pH

The effect of pH of medium on the growth and production of xanthine oxidase activity by *B. pumilus* RL-2d is shown in Fig. 2d. The growth of *B. pumilus* RL-2d varies with the change in pH i.e. 5.0–11.0. Low pH (5.0–6.5) of medium had negative effect on growth and production of xanthine oxidase activity from the organism. Although at pH 7.5, the growth of *B. pumilus* RL-2d is slightly less than that at pH 7.0 but produced higher xanthine oxidase activity i.e. 0.148 U/mg dcw as compared to 0.132 U/mg dcw at pH 7.0.

Temperature

The *B. pumilus* RL-2d was cultured at different temperatures ranging from 40 to 70 °C and xanthine oxidase activity was assayed. It has been observed that there was an increase in the xanthine oxidase activity as well as in biomass production as the incubation temperature increased up to 55 °C (Fig. 2e). *B. pumilus* RL-2d exhibited highest xanthine oxidase activity at 55 °C (0.168 U/mg dcw) with higher yield of biomass (2.4 mg/ml). Beyond this i.e. at 60 °C, there was decrease in growth as well as in xanthine oxidase activity which may be due to denaturation of biological components.

Age of Inoculum

The maximum enzyme activity (0.174 U/mg dcw) was recorded with 28 h old inoculum (Fig. 2f). As the age of inoculum

increased above 28 h, there was slight decrease in the production of xanthine oxidase activity by *B. pumilus* RL-2d.

Incubation Time

The result displayed in Fig. 3 indicates the production of xanthine oxidase activity by *B. pumilus* RL-2d which is related to the biomass production of the organism. The production of xanthine oxidase activity increased in the exponential phase of growth of *B. pumilus* RL-2d, reached to its maximum value (0.186 U/mg dcw) at 24 h of incubation and after that there was a decline in the enzyme production.

Optimization of Reaction Conditions for Assay of Xanthine Oxidase Activity of *B. pumilus* RL-2d

Buffer System and pH

The maximum activity of xanthine oxidase was recorded at pH 7.5 in Tris–HCl buffer (0.184 U/mg dcw) (Fig. 4a).

Temperature

The maximum activity was recorded at 75 °C (0.209 U/mg dcw) (Fig. 4b). As the reaction temperature increased beyond 75 °C, the xanthine oxidase activity declined.

Thermostability of Enzyme

The xanthine oxidase of *B. pumilus* RL-2d was quite stable at 50–70 °C. Based on the first-order enzyme deactivation assumption ($d[E]/dt = -k_d \times [E]$), the xanthine oxidase of *B. pumilus* RL-2d deactivation rate coefficient k_d at 40, 50, 60, 70, 80, 90 °C were 9.1×10^{-6} , 4.5×10^{-6} , 9.5×10^{-6} , 3.3×10^{-5} , 1.7×10^{-4} , $2.3 \times 10^{-4} \text{ s}^{-1}$ and its corresponding half-lives $t_{1/2}$ ($t_{1/2} = k_d/0.693$) were 20, 15, 10, 5.7, 1, 0.8 h, respectively (Fig. 4c).

Effect of Metal Ions and Inhibitors

The effect of various metal ions and inhibitors on the xanthine oxidase activity using resting cells of *B. pumilus*

Fig. 2 Optimization of culture parameters for production of xanthine oxidase by *B. pumilus* RL-2d. **a** Effect of carbon sources, **b** effect of nitrogen sources, **c** effect of yeast extract concentration, **d** effect of pH, **e** effect of temperature, **f** effect of inoculum age

RL-2d has been depicted in Fig. 4d. The activity of xanthine oxidase of this organism was strongly inhibited by AgNO_3 , HgCl_2 and allopurinol to the extent of 95, 85 and 96 %, respectively. The addition of 1 mM CuSO_4 and

CaCl_2 also inhibited the xanthine oxidase activity by 76 and 83 %, respectively. There was a slight inhibition of xanthine oxidase activity in the presence of chelating (EDTA) and reducing (DTT) agents.

Fig. 2 continued

Substrate Concentration

The effect of substrate concentration on the xanthine oxidase activity of *B. pumilus* RL-2d was studied, and

maximum activity (0.206 U/mg dcw) was observed at 0.6 mM of xanthine. Increase in activity was recorded up to 0.6 mM and thereafter it remained more or less same up to 1.0 mM (Fig. 4e).

Fig. 3 Time course for xanthine oxidase activity production and growth of *B. pumilus* RL-2d

Discussion

The enzyme linked processes are important in the synthesis of chemicals and intermediates due to their operation under milder conditions. The present study was focused on the isolation and screening of bacteria from thermal springs for thermostable xanthine oxidase activity. Thermo-alkali tolerant xylanase producing bacteria had also been reported from thermal springs of Manikaran [12]. After exhaustive screening, a highly promising isolate was obtained which has excellent xanthine oxidase activity far more than hitherto reported bacteria [2]. This isolate resembled phenotypically with earlier reported *Bacillus pumilus* [13]. *B. pumilus* RL-2d showed highest xanthine oxidase activity in nutrient broth supplemented with xanthine. Glycerol emerged out to be very suitable source of carbon for maximum enzyme and biomass production by *B. pumilus* RL-2d, while for *Arthrobacter* sp. glucose has been reported as a suitable carbon source for maximum xanthine oxidase production (0.015 U/mg cell) [2]. In the presence of glucose and sucrose, *B. pumilus* RL-2d yielded good biomass but the xanthine oxidase production was less. Sodium succinate and lactose resulted into poor growth *vis-a-vis* less xanthine oxidase activity. The addition of yeast extract in the medium resulted in higher production of xanthine oxidase activity by *B. pumilus* RL-2d. Similar observations were reported by Xin et al. [2] using yeast extract as nitrogen source. After the optimization of carbon and nitrogen source 2.8 fold increase (0.051–0.143 U/mg dcw) in xanthine oxidase activity of *B. pumilus* RL-2d was observed while 5.66-fold increase (0.006–0.034 U/mg cell) in xanthine oxidase activity of *Arthrobacter* sp. has been reported [2]. Increase in xanthine oxidase production was noticed when 20–28 h old preculture was used. However, with increase in the incubation time of preculture beyond 28 h, an increase in cell mass production without enhancement in xanthine

oxidase activity was observed. Tanigaki et al. [6] reported that *Arthrobacter luteus* produced thermostable xanthine oxidase enzyme between pH range 6.0 to 9.0. In the present study, *B. pumilus* RL-2d efficiently grew and produced xanthine oxidase activity around neutral pH (7.5). The optimum temperature for the xanthine oxidase production by *B. pumilus* RL-2d was 55 °C. However, most of xanthine oxidase producing microorganisms reported are mesophilic ones [2, 4, 6, 14, 15]. Dwivedi et al. [16] have isolated a thermophilic organism *Thermus parvatiensis* from Manikaran hot spring having the optimum temperature of 70 °C for growth. The xanthine oxidase production in *B. pumilus* RL-2d was observed between late log and early stationary phase of growth which started after 20 h of incubation and maximum xanthine oxidase production was achieved after 24 h. *Arthrobacter* sp. has been reported to produce xanthine oxidase after 45 h of incubation [2]. *B. pumilus* RL-2d showed maximum xanthine oxidase activity with Tris–HCl buffer of 0.05 M at pH 7.5. Similarly Tris–HCl buffer has been used by Aggarwal and Banerjee during screening of xanthine oxidase producing bacteria from soil [4]. The optimization of culture and reaction conditions based on one variable at a time approach resulted into 4.1-fold increase in xanthine oxidase activity. The xanthine oxidase of *B. pumilus* RL-2d was stable up to 5 h at 70 °C while majority of the reported xanthine oxidases rapidly lose activity at temperature 60–80 °C. Xanthine oxidase of *Arthrobacter* sp. exposed to 0.5 h at 60, 70 and 80 °C could retain 50, 30 %, and <5 % activity respectively [2].

The studies on the inhibition of xanthine oxidase by free metal ions are limited. The inhibition of xanthine oxidase of *B. pumilus* RL-2d by Hg^{2+} , Cu^{2+} , and Ag^{+} ions is similar to as observed earlier with the bovine xanthine oxidase by Mondal et al. [17], suggesting that the interaction of metal ions affects the oxidative half reaction which might be the main cause of the inhibition of xanthine oxidase activity and

Fig. 4 Optimization of reaction parameters for xanthine oxidase activity of *B. pumilus* RL-2d, **a** effect of buffer pH, **b** effect of reaction temperature, **c** stability profile of xanthine oxidase of *B. pumilus* RL-2d at various temperatures, **d** effect of metal ions and inhibitors, **e** effect of substrate concentration

also the interactions between enzyme and metal ions were found to block the terminal sulfur atom of the Mo = S moiety [18]. To our knowledge, this is the first reported

xanthine oxidase from *B. pumilus* RL-2d having high thermal stability. The thermal stability profile of the present xanthine oxidase make it a suitable candidate for potential

Fig. 4 continued

applications for the assay of superoxide dismutase, purines, phosphates of blood serum, nucleotidase and in the food industry for assessment of freshness of meat.

Conclusion

The present study reports a thermostable xanthine oxidase from *B. pumilus* RL-2d that exhibits highest activity and thermal stability among the hitherto reported xanthine oxidases. Since thermostability is regarded as an index of overall biocatalyst stability, higher stability of *B. pumilus* RL-2d xanthine oxidase under operational condition ($t_{1/2}$ 5 h at 70 °C) implies that the enzyme can be used repeatedly for long term biocatalytic reaction which makes it a potential candidate for industrial applications under moderate-to-high temperature conditions.

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Compliance with Ethical Standards

Conflict of interest Authors declare that there is no conflict of interest.

References

1. Apaya KL, Chichioco-Hernandez CL (2011) Xanthine oxidase inhibition of selected Philippine medicinal plants. *J Med Plants Res* 5:289–292
2. Xin Y, Yang H, Xia X, Zhang L, Zhang Y, Cheng C, Wang W (2012) Expression, purification and partial characterization of a xanthine oxidase (XOD) in *Arthrobacter* sp. *Process Biochem* 47:1539–1544. doi:[10.1016/j.procbio.2012.01.007](https://doi.org/10.1016/j.procbio.2012.01.007)
3. Papapostolou I, Georgiou CD (2010) Superoxide radical is involved in the sclerotial differentiation of filaments phytopathogenic fungi: identification of a fungal xanthine oxidase. *Fungal Biol* 114:387–395. doi:[10.1016/j.funbio.2010.01.010](https://doi.org/10.1016/j.funbio.2010.01.010)
4. Agarwal A, Banerjee UC (2009) Screening of xanthine oxidase producing microorganisms using nitroblue tetrazolium based colorimetric assay method. *Open Biotechnol J* 3:46–49. doi:[10.2174/1874-0707/09](https://doi.org/10.2174/1874-0707/09)

5. George J, Struthers AD (2008) The role of urate and xanthine oxidase inhibitors in cardiovascular disease. *Cardiovasc Ther* 26:59–64. doi:[10.1111/j.1527-3466.2007.00029](https://doi.org/10.1111/j.1527-3466.2007.00029)
6. Tanigaki N, Furukawa K, Sogabe Y, Emi S (1993) Thermostable xanthine oxidase from *Arthrobacter luteus*. United States Patent 518525
7. Pauff JM, Zhang J, Bell CE, Hille R (2008) Substrate orientation in xanthine oxidase: crystal structure of enzyme in reaction with 2-hydroxy-6-methylpurine. *J Biol Chem* 283:4818–4824. doi:[10.1074/jbc.M707918200](https://doi.org/10.1074/jbc.M707918200)
8. Zhang Y, Xin Y, Yang H, Zhang L, Xia X, Tong Y, Chen Y, Ma L, Wang W (2012) Novel affinity purification of xanthine oxidase from *Arthrobacter* M3. *J Chromatogr B* 906:19–24. doi:[10.1016/j.jchromb.2012.08.007](https://doi.org/10.1016/j.jchromb.2012.08.007)
9. Dolmeci N, Cete S, Arslan F, Yaşar A (2012) An amperometric biosensor for fish freshness detection from xanthine oxidase immobilized in polypyrrole polyvinylsulphonate film. *Artif Cells Nanomed Biotechnol* 40:275–279. doi:[10.3109/10731199.2011.646410](https://doi.org/10.3109/10731199.2011.646410)
10. Sathisha KR, Khanum SA, Chandra JNNS, Ayisha F, Balaji S, Marathe GK, Gopal S, Rangappa KS (2011) Synthesis and xanthine oxidase inhibitory activity of 7-methyl-2-(phenoxymethyl)-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one derivatives. *Bioorg Med Chem* 19:211–220. doi:[10.1016/j.bmc.2010.11.034](https://doi.org/10.1016/j.bmc.2010.11.034)
11. Kalra S, Jena G, Tikoo K, Mukhopadhyay AK (2007) Preferential inhibition of xanthine oxidase by 2-amino-6-hydroxy-8-mercaptapurine and 2-amino-6-purine thiol. *BMC Biochem* 8:1–11. doi:[10.1186/1471-2091-8-8](https://doi.org/10.1186/1471-2091-8-8)
12. Singh AK, Tripathi BM, Sahay H, Singh RN, Kaushik R, Saxena AK, Arora DK (2010) Biochemical and molecular characterization of thermo-alkali tolerant xylanase producing bacteria from thermal springs of Manikaran. *Indian J Microbiol* 50:S2–S9. doi:[10.1007/s12088-010-0071-4](https://doi.org/10.1007/s12088-010-0071-4)
13. Kumar D, Verma R, Sharma P, Rana A, Sharma R, Prakash C, Bhalla TC (2010) Production and partial purification of xylanase from a new thermophilic isolate. *Biol Forum Int J* 2:83–87
14. Mahajan J, Kumar R, Kaur R, Arora S (2012) Exploration of xanthine oxidase producing microorganisms from hypoxanthine supplemented soil and their inhibitors from plants employing nitrobluetetrazolium based colorimetric assay method. *GARJEST* 1:010–013
15. Nakanishi T, Machida Y (1982) Method and test composition for the determination of the substrate for xanthine oxidase. United States Patent 4341868A
16. Dwivedi V, Kumari K, Gupta SK, Kumari R, Tripathi C, Lata P, Niharika N, Singh AK, Kumar R, Nigam A, Garg N, Lal R (2015) *Thermus parvatiensis* RL^T sp. Nov, isolated from a hot water spring, located atop the Himalayan ranges at Manikaran, India. *Indian J Microbiol*. doi:[10.1007/s12088-015-0538-4](https://doi.org/10.1007/s12088-015-0538-4)
17. Mondal MS, Sau AK, Mitra S (2000) Mechanism of the inhibition of milk xanthine oxidase activity by metal ions: a transient kinetic study. *Biochim Biophys Acta* 1480:302–310. doi:[10.1016/S0167-4838\(00\)00084-4](https://doi.org/10.1016/S0167-4838(00)00084-4)
18. Gardlik G, Rajagopalan KV (1990) The state of reduction of molybdopterin in xanthine oxidase and sulfite oxidase. *J Biol Chem* 265:13047–13054