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Research Article

ISOLATION AND SCREENING OF XANTHINE OXIDASE PRODUCING BACTERIA FROM THERMAL SPRINGS OF HIMACHAL PRADESH

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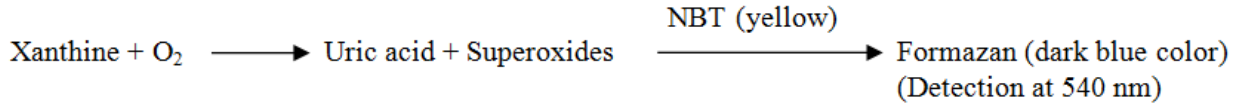
ABSTRACT
Xanthine oxidase is a highly versatile and ubiquitous complex molybdoflavoprotein and has catalytic role in purine degradation, metabolizing hypoxanthine and xanthine to uric acid with the generation of superoxides. In the present study, Nitrobluetetrazolium (NBT) based colorimetric assay was used to screen the xanthine oxidase producing microorganisms from the soil and water samples of thermal springs of Himachal Pradesh, India. A total of fortyisolates were obtained on primary screening of xanthine oxidase producing microorganisms. The hyper-active bacterial isolate RL-2d, isolated during the screening has been identified by physiological and biochemical tests as *Bacillus* sp. and named *Bacillus* sp. RL-2d. The present study showed that *Bacillus* sp.RL-2d exhibited highest xanthine oxidase activity among all the isolates screened for xanthine oxidase.

Keywords: *Nitrobluetetrazolium, Thermal Springs, Xanthine, Screening, Microbial Xanthine Oxidase*

INTRODUCTION
Xanthine oxidase is a complex molybedo-flavo enzyme whichcatalyzes the oxidation of hypoxanthine with oxygen to xanthine and lastly to uric acid (Zhang *et al.,* 2012). Mammalian xanthine oxidase initially exists as a dehydrogenase form and it can be easily converted to oxidase form either irreversibly or reversibly (Della and Stripe, 1972; Enroth *et al.,* 2000; Nishino and Nishino, 1997). This enzyme was first isolated from cow's milk as a major component of the milk fat globule membrane (Xin *et al.,* 2012). The different forms of xanthine oxidoreductases are generally found in living organisms, such asarchaea, bacteria, fungi, plants, and metazoans and these isoforms from different sources are all fundamentally similar with regard to molecular properties, prosthetic group content and substrate specificity (Agarwal and Banerjee, 2009).
This enzyme has also been reported from various organs of animals i.e. liver, brain, gastrointestinal tract and cardiovascular tissues (George and Struthers, 2008). The enzyme from various sources are proteins of molecular weight of approximately 300 kDa having two identical and catalytically independent subunits and each subunit contains one molybdenum center, two iron sulfur (2Fe-2S) centers and flavin adenine dinucleotide (Hille and Nishino, 1995). Xanthine oxidase has been extensively used in clinical assay for estimation of inorganic phosphate, adenosine deaminase and 5'-nucleotidase mainly in the diagnosisof liver diseases. Moreover, xanthine oxidase/xanthine dehydrogenase has also been playing vital roles in innate immune system, cardiovascular diseases and employed as antimicrobial agents (Vorbach *et al.,* 2003; Berry and Hare, 2004; Martin *et al.,* 2004).
Xanthine oxidase plays a vital role in the oxidation of purines, hydroxylation of pterines, aromatic heterocycles, aliphatic and aromatic aldehydes and also shows the detoxification or activation of endogenous compounds and xenobiotics (Borges *et al.,* 2002). The xanthine oxidase is also used in the detection of superoxide dismutase and plays a role in the food industries for assessment of freshness of meat (Goldstein *et al.,* 1988).
In the present study, nitrobluetetrazolium (NBT) based colorimetric assay is used to screen the xanthine oxidase producing microorganisms from the soil and water samples obtained from thermal springs of Himachal Pradesh. The basic principle of assay is that the superoxides produced by microbial cultures, grown in xanthine rich medium interacts with nitrobluetetrazolium (NBT) solution and produces dark blue color formazan, showing the presence of xanthine oxidase (Agarwal and Banerjee, 2009). The reaction is as follows:



MATERIALS AND METHODS

Chemicals

All the chemicals were of analytical grade. The xanthine, allopurinol and media components were purchased from Hi Media (Mumbai; India).

Sample Collection

The soil and water samples were collected from different hot springs of Himachal Pradesh, India viz. Manikaran hot springs (80 °C-95 °C), Kheer Ganga hot springs (40 °C-55 °C), Tatapani hot springs (40 °C-65 °C) and Vashishtha hot springs (50 °C-55 °C) for the isolation of thermophilic bacteria for xanthine oxidase activity. All the water/soil samples from hot springs had pH in the range of 6.0 to 8.0.

Isolation of Bacteria for Xanthine Oxidase Activity

One gram soil or 1 ml water samples were used for the isolation of xanthine oxidase producing bacteria and the samples were added into a 50 ml of mineral salt medium (MSM) supplemented with 1mM l^{-1} xanthine as sole source of carbon and nitrogen. The MSM broth contained following in g/l of deionised water: $Na_2HPO_4.12H_2O$, 2.5; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.03; $CaCl_2.2H_2O$, 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.One ml of the culture was transferred to 50 ml of fresh medium and incubated under similar conditions and 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^{-1}). Morphologically distinct colonies appeared on the plates of MSM agar supplemented with xanthine after incubation at 55°C for 48 h, which were streaked onto the fresh selective medium (containing xanthine) to obtaina pure culture. 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 Xanthine Oxidase Producing Bacterial Isolates

The bacterial colonies growing on MSM plates supplemented with xanthine (1 mM l^{-1}) were further selected for shake flask studies. A seed culture containing nutrient broth medium was prepared by inoculating a single colony of the bacterial isolate into 250 ml Erlenmeyer flask and incubated under shaking conditions (150 rpm) at 55 °C for 24 h.Two percent (v/v) of seed culture was transferred to a 250 ml flask containing 49 ml of production medium havingnutrient broth with required xanthine concentration. The flasks were incubated at 55 °C under shaking conditions (150 rpm) for 24 h. Cells were harvested by centrifugation at 12,000 g for 5 min at 4 °C and the cells were washed thrice with 0.05 M Tris-HCl buffer (pH 7.5). The washed cells were 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.

Morphological, Biochemical and Physiological Characterization

The morphological, physiological and biochemical tests were carried at Institute of Microbial Technology (IMTECH), Chandigarh, India. The different tests such as Gram staining reaction, spore position, density, pigmentation and shape, growth on MacConkey agar, aerobic or anaerobic growth, nitrate reduction, H_2S production and arginine dihydrolysis were performed for the identification of efficient xanthine oxidase producing thermophilic bacterial isolate, RL-2d. 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. The various physiological tests such as growth on different temperature (4, 10, 15, 25, 30, 37, 42, 55 and 60 °C), pH (5.0, 5.7, 6.8, 8.0, 9.0 and 11.0) and different concentration of sodium chloride (NaCl) (2.5, 5.0, 7.0, 8.5 and 10%) were carried out for the characterization of microorganism.

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Detection of Microbial Xanthine Oxidase using the Nitrobluetetrazolium Based Assay

Nitrobluetetrazolium based assay method was used for the detection of xanthine oxidase activity in resting cells (Agarwal and Banerjee, 2009). Each reaction contained 500µl (4mg/ml) of microbial cell lysed with NaOH (1 mol/l) and500µl NBT solution containing 50 mmol/lTris-HCl, pH 7.5, 1mmol/lxanthine and 0.50 mmol/lnitrobluetetrazolium. The reaction mixture was incubated for 5 minutes at 55 ^{o}C, centrifuged and absorbance of supernatant was measured at 540 nm. This was used as a test sample. Two control experiments were set with this assay. First control consists of 500 µl NBT solution and500 µlNaOH (1 mol/l) which is termed as negative control.This was carried out to see the interaction between NBT and xanthine in alkaline conditions. Second control experiment was set in which 500µl microbial culture was incubated with allopurinol (50 mmol/l) for 5 minutes at 55 ^{o}C and to this mixture, 500µl NBT solution was added and again incubated for 5 minutes at ambient temperature,centrifuged and the absorbance of the supernatant was read at 540nm. This control experiment determined the superoxides formed by the action of enzymes other than xanthine oxidase present in the microbial cultures. Positive control was set up with commercially available xanthine oxidase, keeping all the reaction parameters same.

RESULTS AND DISCUSSION

Results

Isolation and Screening of Xanthine Oxidase Production Bacteria

Enrichment culture technique method was used for the isolation of thermostable xanthine oxidase producing bacteria.Forty bacterial strains were isolated from water/soil samples of Tatapani, Manikaran, Kheerganga, and Vashishta thermal springs of Himachal Pradesh, India (Table 1).

Table 1: Xanthine oxidase activity of the bacterial isolates from various thermal springs of Himachal Pradesh

S. No.	Source	Xanthine oxidase activity (U/mg dcw)	S.No.	Source	Xanthine oxidase activity (U/mg dcw)
Tatapani hot spring (TP)			1	21	VS-21	0.006
TP-01		0.008	2	22	VS-22	-
TP-02		-	3	23	VS-23	-
TP-03		-	4	24	VS-24	-
TP-04		-	5	**Kheer Ganga hot spring (KG)**		
TP-05		0.006	6	25	KG-25	0.001
TP-06		-	7	26	KG-26	-
TP-07		-	8	27	KG-27	-
TP-08		-		28	KG-28	-
Manikaran hot spring (MN)			9	29	KG-29	-
MN-09		-	10	**Manikaran hot spring (RL)**		
MN-10		0.018	11	30	RL-30	-
MN-11		-	12	31	RL-31	0.023
MN-12		-	13	32	RL-32	-
MN-13		-	14	33	RL-33	-
MN-14		0.012	15	34	RL-34	-
MN-15		-	16	35	RL-35	-
MN-16		-	17	36	RL-2d	0.036
MN-17		0.009	18	37	RL-37	-
MN-18		-		38	RL-38	0.008
Vashishta hot spring (VS)			19	39	RL-39	-
VS-19		-	20	40	RL-40	0.017
VS-20		0.014				

These thermophilic isolates were assigned code number and were screened for the production of xanthine oxidase activity.After primary screening on MSM agar plates supplemented with xanthine, 12 bacterial cultures were selected for shake flask studies. One isolate, RL-2d was found to possess highest xanthine oxidase activity (0.036 U/mg dcw) among the twelve xanthine oxidase producing isolates and was used for further studies.

Identification of Selected Bacterial Isolate

Identification of efficient xanthine oxidase producing thermophilic bacterial isolate, RL-2d was made by studying the different morphological, physiological and biochemical parameters. Cells of RL-2d were small rods, motile, Gram positive, thermophilic (40 °C-70 °C) and high pH tolerant (6.5-11). The isolate was aerobic in nature, produced acid from dextrose, mannitol, fructose, and trehalose. The isolate was later identified at the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh tentatively as *Bacillus* sp. RL-2d. The morphological and biochemical properties of the isolate are listed in Table 2.

Table 2: Morphological and biochemical characteristics of *Bacillus* sp. RL-2d isolated from Manikaran thermal spring

Morphological Characteristics	
Tests	
Colony morphology	
Configuration	Round
Margin	Entire
Elevations	Flat
Surface	Smooth
Density	Translucent
Pigments	White
Gram's reaction	**(+)**
Shape	Rods
Size Long Arrangement Single	
Spore	**(+)**
Position	Central
Shape	Round
Fluorescence	**(-)**
Motility	**(+)**
Biochemical test	
Growth on MacConkey agar	(-)
Indole test	(-)
Methyl Red Test	(-)
Casein Hydrolysis	(+)
Starch Hydrolysis	(-)
Nitrate Reduction	(+)
H_2S Production	(-)
Catalase Test	(+)
Arginine dihydrolysis	(+)
Physiological TestsGrowth at Temperature4 °C	
(-)	
10 °C	(-)
15 °C	(-)
25 °C	(-)
30 °C	(+)
37 °C	(+)
42 °C	(+)
55 °C	(+)
60 °C	(+)
Growth at pH	
5.0	(-)
5.7	(-)
6.8	(+)
8.0	(+)
9.0	(+)
11.0	(+)
Growth at NaCl (%)	
2.5	(+)
5.0	(-)
7.0	(-)
Acid production from carbohydrates	
Xylose	(-)
Innulin	(-)
Cellibiose	(-)
Dextrose	(+)
Mannose	(-)
Fructose	(+)
Maltose	(-)
Inositol	(-)
Mannitol	(+)
Sorbitol	(-)
Trehalose	(+)

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Detection of Microbial Xanthine Oxidase using NBT

The bacterial isolate showed positive growth on the nutrient agar plates, containingxanthine as a sole source of carbon and nitrogen. The microbial cultures reactwith NBT solution and give dark blue color. There was nocolor developed in the negative control (Figure 1), while the microbial cultures which were treated with allopurinol (standard inhibitor of xanthine oxidase), showed the formation of light blue colourand resulting into the decrease in absorbance. The results ofprimary screeningof forty strains showed that xanthine oxidase activity varied from good to moderate to negligible amount (Table 1).

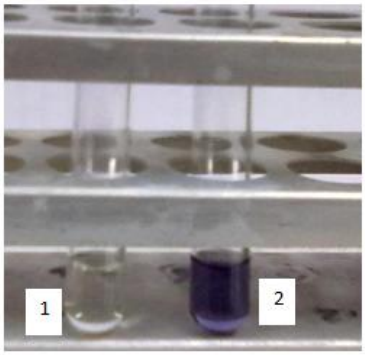

Figure 1: NBT colorimetric assay of xanthine oxidase producing microorganism. 1: negative control, 2: blue color of formazan formed due to the interaction of the super oxides with NBT

Discussion

The present study was focused on the isolation and screening of bacteria for xanthine oxidase activity from thermal springs of Himachal Pradesh, India using NBT assay method. After the comprehensive screening, a highly promising isolate having excellent xanthine oxidase activity which was far more than hitherto reported bacteria (Xin *et al.*, 2012; Agarwal and Banerjee, 2009). Screening of these isolates carried out using NBT assay method gave one better isolate (0.036 U/mg dcw of xanthine oxidase activity) and this isolate was found to be *Bacillus* sp. RL-2d by determination of biochemical and physiological parameters. The superoxides produced during the course of the oxidation of xanthine to uric acid by xanthine oxidase and the interaction of the NBT and superoxides produces dark blue color which appears due to the formation of formazan.

With the increase in the cell mass concentration, the absorbanceincreases whichindicate that the increased amount of formazan formed due to the excess of superoxides produced during the reduction of the oxygen by microbial xanthineoxidase. However, when allopurinol (inhibitor of xanthine oxidase) was added to the reactionmixture, color intensity or the production of formazan decreased due to the decrease inthe production of superoxide radicals; as allopurinol inhibitedthe xanthine oxidase activity and hence free radical formation.Many internal factors likemitochondrial electron chain and autooxidation reactions can be responsible for the production of superoxides which produces the slight blue color on interaction with NBT (Agarwal and Banerjee, 2009). No change in color intensity either in presence of allopurinol or in absence of allopurinol, indicates that these microorganisms do not have the xanthine oxidase activity. In the present study, the NBT assay method is used for the screening of large number of microorganisms which is simple and rapid for determining the xanthine oxidase activity in comparison to previous assays (Woolfolk and Downard, 1978). The screening of bacterial isolate having excellent xanthine oxidase activity make it a suitable candidate for potential applications for the assay of superoxide dismutase, phosphates of blood serum, in the food industry for assessment of freshness of meat andfor the screening of xanthine oxidaseinhibitors.

Conclusion

The present study reports a new xanthine oxidase from *Bacillus* sp.RL-2d that exhibits highest xanthine oxidase activity in comparison to earlier reported bacteria. Xanthine oxidase of this organism has potential for use in developing screening methods for finding xanthine oxidase inhibitors.

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REFERENCES
Agarwal A and Banerjee UC (2009). Screening of xanthine oxidase producing microorganisms using nitrobluetetrazolium based colorimetric assay method. *The Open Biotechnology Journal* **3** 46-49.
Berry CE and Hare JM (2004). Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *The Journal of Physiology* **555**(3) 589-606.
Borges F, Fernandes E and Roleira F (2002). Progress towards the discovery of xanthine oxidase inhibitors. *Current Medicinal Chemistry* **9** 195-217.
Della CE and Stripe F (1972). The regulation of rat lever xanthine oxidase: involvement of thiol groups in the conversion of the enzymatic activity from dehydrogenase into oxidase and purification of the enzyme. *Biochemical Journal* **126** 739-45.
Enroth C, Eger BT, Okamoto K, Nishino T, Nishino T and Pai EF (2000). Crystal structure of bovine milk xanthine dehydrogenase and xanthine oxidase: structure based mechanism of conversion. *Biochemistry* **97**(20) 10723-28.
George J and Struthers AD (2008). The role of urate and xanthine oxidase inhibitors in cardiovascular disease. *Cardiovascular Therapeutics* **26** 59–64.
Goldstein S, Michel C, Bors W, Saran M and Czapski GA (1988). A critical reevaluation of some assay methods for superoxide dismutase activity. *Free Radical Biology and Medicine* **4** 295-303.
Hille R and Nishino T (1995). Xanthine oxidase and xanthine dehydrogenase. *The FASEB Journal* **9** 995-1003.
Martin HM, Hancock JT, Salisbury V and Harrison R (2004). Role of xanthine oxidoreductase as an antimicrobial agent. *Infection and Immunity* **72**(9) 4933-4939.
Nishino T and Nishino T (1997). The conversion from the dehydrogenase type to the oxidase type of rat liver xanthine dehydrogenase by modification of cysteine residues with fluorodinitrobenzene. *Journal of Biological Chemistry* **272** 29859-64.
Vorbach C, Roger H and Capecchi MR (2003). Xanthine oxidoreductase in central to the evolution and function of the innate immune system. *Trends in Immunology* **24**(9) 512-517.
Woolfolk CA and Downard JS (1978). Bacterial xanthine oxidase from *Arthrobacter* S-2. *Journal of Bacteriology* **135** 422–428.
Xin Y, Yang H, Xia X, Zhang L, Zhang Y, Cheng C and Wang W (2012). Expression, purification and partial characterization of a xanthine oxidase (XOD) in *Arthrobacter* sp. *Process Biochemistry* **47** 1539-1544.
Zhang Y, Xin Y, Yang H, Zhang L, Xia X, Tong Y, Chen Y, Ma L and Wang W (2012). Novel affinity purification of xanthine oxidase from *Arthrobacter* M3. *Journal of Chromatography B* **906** 19–24.