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Medium optimization for extracellular urate oxidase production by a newly isolated *Aspergillus Niger*

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Abstract--Urate oxidase is a peroxisomal enzyme with four equal subunits that convert uric acid to allantoin, a more soluble metabolite for excretion. The usage of uricase as a drug in medicine is to treat hyperuricemia. Many microorganisms have been used for uricase production such as *Streptomyces exfoliates*, *Pseudomonas aeruginosa*, and *Aspergillus flavus*. In this study, soil samples were collected and then cultured in a screening medium including uric acid as the sole carbon source. Samples with the higher ability of uricase production were selected for enzyme assay. Enzyme activity was measured by spectrophotometry and the sample with the maximal uricase activity was identified as *Aspergillus niger* and selected for further studies. According to the results of experiments, the optimized temperature for enzyme production by *Aspergillus niger* was determined to be 35±2°C.

The best carbon and nitrogen source was glucose and NH_4NO_3 , and the highest enzyme activity was observed in the presence of Cu^{2+} ion.

Keywords---*Uricase; urate oxidase; optimization; Aspergillus niger.*

Introduction

Uricase (urate oxidase EC 1.7.3.3) is a peroxisomal homotetrameric enzyme with four equal subunits that catalyze the conversion of uric acid to allantoin, a more soluble metabolite for excretion [1] [2-4]. The enzymatic reaction [5] is as follows (figure 1):

The reaction of uric acid, O_2 , and H_2O is catalyzed by uricase to form 5-hydroxyisourate, which is unstable and transforms to 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline. Then this product decarboxylates and forms allantoin [1]. The usage of uricase as a drug in medicine is to treat hyperuricemia, so it can use in gout and TLS [6]. Another usage of uricase is in diagnostic kits as a reagent for enzymatic determination of uric acid [7]. It is used with peroxidase and 4-aminoantipyrine. Indeed, peroxidase catalyzes the reaction of 4-aminoantipyrine and H_2O_2 , which is the result of the uricase reaction, and a pink color product is formed [8]. Uricase enzyme has been isolated from different microbial, plant, and animal sources. Microorganisms such as bacteria and fungi normally have uricase enzymes [7], so they are used for uricase production. Some of the microorganisms used to produce uricase are *Streptomyces exfoliatus* [9], *Pseudomonas aeruginosa* [7], *Bacillus subtilis* [10], *Candida tropicalis* [11], and *Aspergillus flavus* [8]. Wheat, chickpea, and broad bean are plant sources, which were studied for uricase production and isolation [5]. Animals like birds, some species of reptiles, some primates, and most vertebrates have uricase in their liver [12]. Porcine and fish are animal sources that were studied for uricase purification [5]. However, higher primates and humans have lost uricase due to evolutionary changes [7]. Because of this mutation, uric acid is not converted to allantoin in the human body. This condition has both advantages and disadvantages. Uric acid is a potent antioxidant and is a part of the body's protective system against oxygen radicals [13]. Though, if uric acid reaches serum levels above 6.8 mg/dl, hyperuricemia happens [14] and gout and renal failure may happen secondary to hyperuricemia, and the risk of tumor lysis hyperuricemia may also increase after chemotherapy [6]. So, now studies have focused on the usage of external uricase isolated from different sources, for the management of hyperuricemia [5]. Pegloticase or Puricase is a PEG-modified recombinant mammalian uricase, that has been approved by the FDA for treatment of conventional therapy refractory gout [15]. Rasburicase is also a recombinant uricase that has been expressed in *Saccharomyces cerevisiae* [16]. Rasburicase has been approved for TLS [1].

Uricase has some appropriate (e.g., high efficacy and selectivity of catalytic reaction on the substrate) and inappropriate (e.g., short in vivo half-life, limited action effect, low catalytic activity at physiological temperature and pH) characterizations, like other therapeutic enzymes [17]. Temperature stability of uricases is at the range of 10-100 °C, based on the source of the enzyme [10, 18-

[24]. The optimum temperatures for uricase function are different based on the source of enzyme and at the range of 25-50 °C [18, 25-29]. The general stability of the enzyme is also different due to the source of the enzyme. Several studies have been accomplished to improve enzyme stability and catalytic activity [17]. Studies on the uricase of *Bacillus fastidious* [30], *Glycine max* [31], *Arthrobacter globiformis* [32], and *Aspergillus flavus* [33] have shown that uricase is a cofactor-independent enzyme. Many microorganisms have been used for uricase production yet, but according to their significant clinical uses, it is important to find new and economical sources for enzyme production [34]. In this paper, microorganisms with higher uricase production ability were isolated and optimization of the cultural and environmental conditions for increasing uricase production was studied in the highest producer.

Material and Methods

Sample collection, media, and cultivation

Soil samples were collected from different districts of Urmia (West Azarbaijan province, northwest of Iran). First, 2 gr of each sample was dissolved in 15 ml of sterile distilled water. After 2 minutes, 100 µL of supernatant of each sample was transferred to LB broth as the enriched medium, and then incubated for 24 h 37°C. Screening medium containing 0.4% NH₄Cl, 0.4% NaNO₃, 0.5% KH₂PO₄, 0.1% Glucose, 0.3% uric acid and 1.5% microbiological agar was prepared and pH was adjusted to 6.5±0.2. Also, the broth medium (without agar) was prepared. Samples were transferred to these media and then incubated at 37°C. Samples that produced more uricase (according to the size of the clear zone on the plate of screening medium) were selected for further study.

Uricase assay

After 24 h samples were discharged from the incubator and an enzyme assay was done for evaluating the quantity of uricase production and the activity of the produced enzyme. First, the standard reaction mixture containing 0.6 ml of 2mM uric acid dissolved in 0.1 M sodium borate buffer (pH 8.5), 0.15 ml of 30 mM 4-aminoantipyrine, 0.1 ml of 1.5% phenol, 0.05 ml of 15 U/ml horseradish peroxidase, and 0.1 ml of enzyme solution was prepared and incubated at 25°C for 20 min. then, 1 ml of ethanol was added to each sample to terminate the reaction. Samples with the higher ability of uricase production produced stronger red color and enzyme activity was measured at 540 nm by spectrophotometry[35]. The enzyme assay was done every day until seven days. The best result was observed on the 7th day. The sample with the highest activity was selected for the rest of the studies.

Identifying the selected sample

The selected sample was a fungus (based on the appearance of colonies), so it was cultured in sabouraud dextrose agar medium. After complete growth on medium, a slide of it was prepared and sent to a mycologist to identify.

Effect of different carbon and nitrogen sources on uricase production

For studying the effect of different carbon sources, glucose, sucrose, lactose, sodium acetate, and citric acid were used separately with a concentration of 0.1% in the screening medium.

The effect of different nitrogen sources was also studied with 0.4% of each nitrogen source (NH_4NO_3 , peptone, glycine, and yeast extract) in the screening medium.

Samples were incubated at 37°C for 7 days and then enzyme assay was done.

Effect of metal ions on uricase production

For studying the effect of metal ions, Cu^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} and Fe^{2+} were added separately to medium, with a concentration of 0.001 M. After 7 days of incubation at 37°C, enzyme assay was done.

Effect of temperature on uricase production

The effect of temperature on uricase activity was determined by incubating the sample at different temperatures (6 ± 2 °C, 25 ± 2 °C, and 37 ± 2 °C). Samples were incubated for 7 days and then enzyme assay was done.

Results

Isolation and identification of microorganisms

40 soil samples were collected from different districts of Urmia (West Azarbaijan province, northwest of Iran) to isolate the best uricase-producing microorganism. Microorganisms with the uricase production ability made clear zones on the screening medium, which indicate the conversion of uric acid to allantoin in the presence of uricase. Microorganisms that made bigger clear zones on solid media, was selected and transferred to the broth media. Then uricase assay was done and the activity of samples was measured by spectrophotometry. Samples with the ability to produce uricase in broth media, degraded uric acid results in color change. The microorganism with the highest activity at assay was identified as *Aspergillus niger* based on macroscopy and microscopy recognition. It was chosen for the rest of the tests.

Effect of different carbon and nitrogen sources on uricase production

Results of enzyme assay-based activity at 540 nm have shown in figure 2, considering STDEV of results. According to the results, glucose was the best carbon source for uricase production by *Aspergillus niger*. It also produced the highest red color at the enzyme assay. So the carbon sources on the elevation of uricase activity followed a glucose > sucrose > sodium acetate > lactose > citric acid sequence. The clear zone on the plates of the screening medium which are different at the carbon source has shown in figure 3.

Also, the results of the enzyme assay based on difference at nitrogen source has shown in figure 4 with counting STDEV of results. According to these results, NH_4NO_3 was determined as the best nitrogen source for uricase production by *Aspergillus niger*, based on the highest activity at 540 nm as the result of the enzyme assay. It also produced darker red color at enzyme assay. So the nitrogen sources on the elevation of uricase activity followed an $\text{NH}_4\text{NO}_3 > \text{yeast extract} > \text{peptone} > \text{glycine}$ sequence. The clear zone on the plates of the screening medium which are different at the nitrogen source has shown in figure 5.

Effect of metal ions on uricase production

To increase the amount of uricase produced by *Aspergillus niger*, some metal ions were added to the broth media. According to the results of enzyme assay based on activity at 540 nm (figure 6), Cu^{2+} was determined as the best additive ion for uricase production by *Aspergillus niger*. It also showed more red color at enzyme assay. So the additive metal ions on the promotion of uricase activity followed a $\text{Cu}^{2+} > \text{Mn}^{2+} > \text{Zn}^{2+} > \text{Mg}^{2+} > \text{Fe}^{2+}$ sequence. The clear zone on the plates of the screening medium which are different at additive ions has shown in figure 7.

Effect of temperature on uricase production

According to the results of the assay based on enzyme activity at 540 nm (figure 8) with considering STDEV of results, and intensity of producing red color, $35 \pm 2^\circ\text{C}$ was determined as optimum temperature for uricase production by *Aspergillus niger*. The clear zone on the plates of the screening medium at different temperatures has shown in figure 9.

Discussion

These days many fungal sources have been used for uricase production such as *Candida tropicalis* [11], *Aspergillus flavus* [36], and *Candida utilis* [37]. In this study, 40 soil samples were collected and microorganisms with uricase production ability were isolated for studying optimized conditions for increasing uricase production. Uricase assay showed that *Aspergillus niger* is the best choice for uricase production. Then the effect of four factors on uricase production including carbon source, nitrogen source, metal ions, and temperature was studied. Cause uricase enzyme has copper in structure as a cofactor [38], Cu^{2+} was added to each sample (except samples with additive ions) at a concentration of 10^{-3} M to increase uricase production like a cofactor. In 2011, an optimized condition for the activity of uricase isolated from *Pseudomonas aeruginosa* was studied. The optimized temperature for maximum activity was determined to be 35°C . Ca^{2+} increased enzyme activity, whereas Cu^{2+} , Mg^{2+} , Zn^{2+} , Co^{2+} and Fe^{2+} reduced enzyme activity [39]. According to a study on the optimized condition for uricase production by *Aspergillus flavus* in 2002, sucrose was determined to be the best carbon source, and uric acid was identified as the best nitrogen source for uricase production and activity [40]. In 2013, optimized conditions for uricase production by two strains of *Aspergillus niger* were studied. According to this study, sucrose and starch were determined as the best carbon sources, and casein and peptone were selected as the best nitrogen sources, and higher temperatures (45°C and 35°C) were determined as the best temperature for

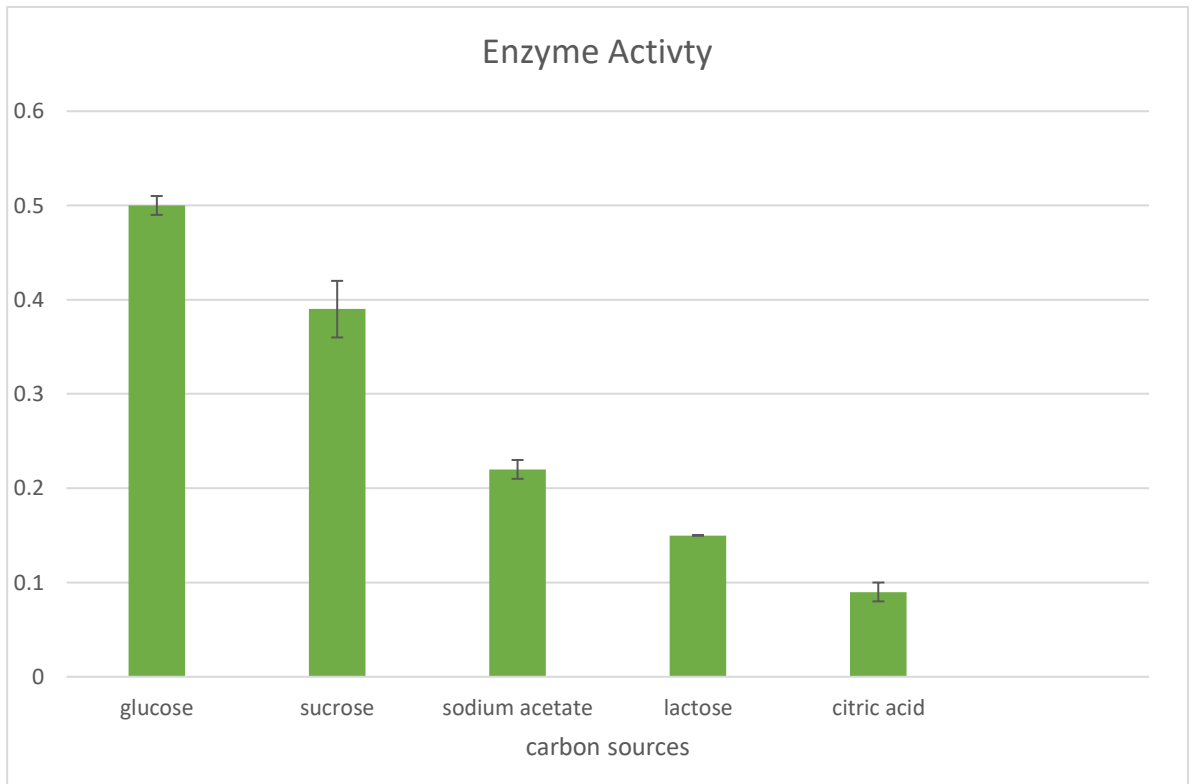


Figure 2. Effect of different carbon sources on uricase activity

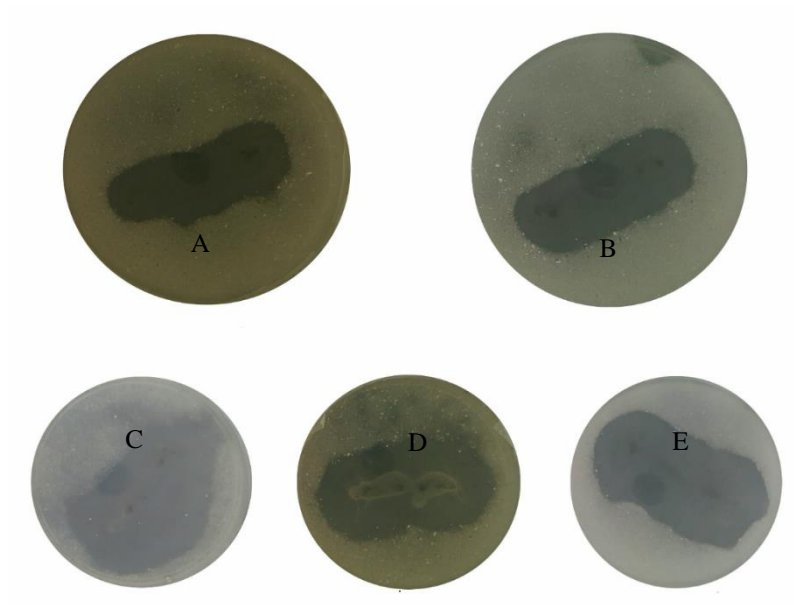


Figure 3. *Aspergillus niger* on uric acid plates with different carbon sources. A) Glucose B) Sucrose C) Sodium acetate D) Lactose E) Citric acid

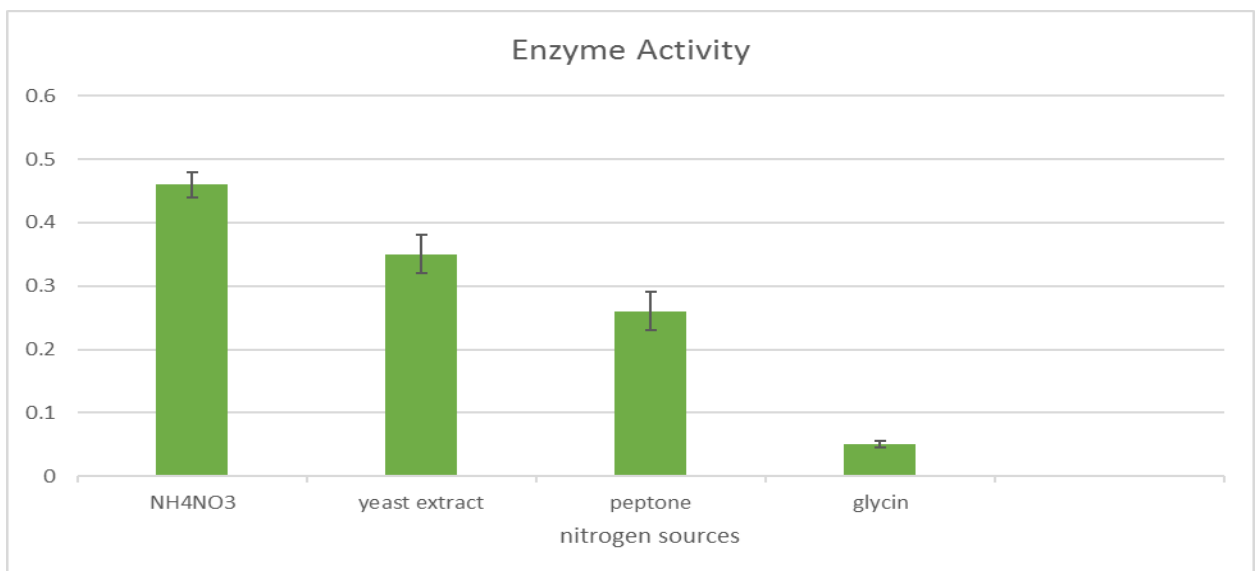


Figure 4. Effect of different nitrogen sources on uricase activity

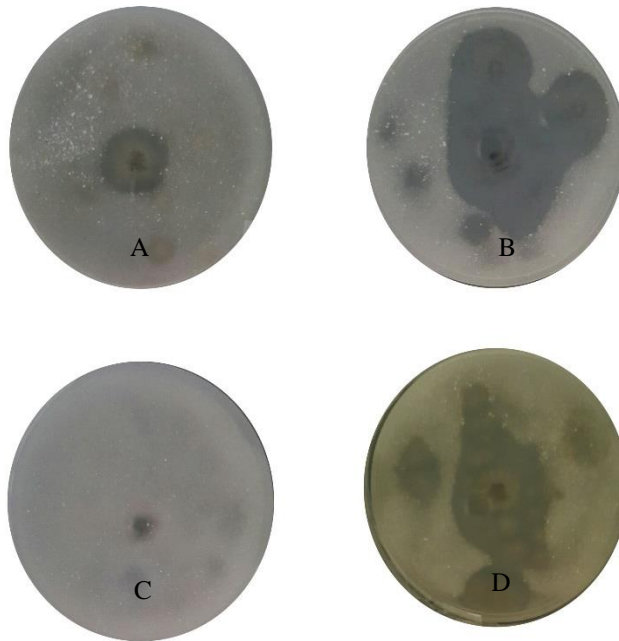


Figure 5. *Aspergillus niger* on uric acid plates with different nitrogen sources. A) NH₄NO₃ B) Yeast extract C) Glycine D) Peptone

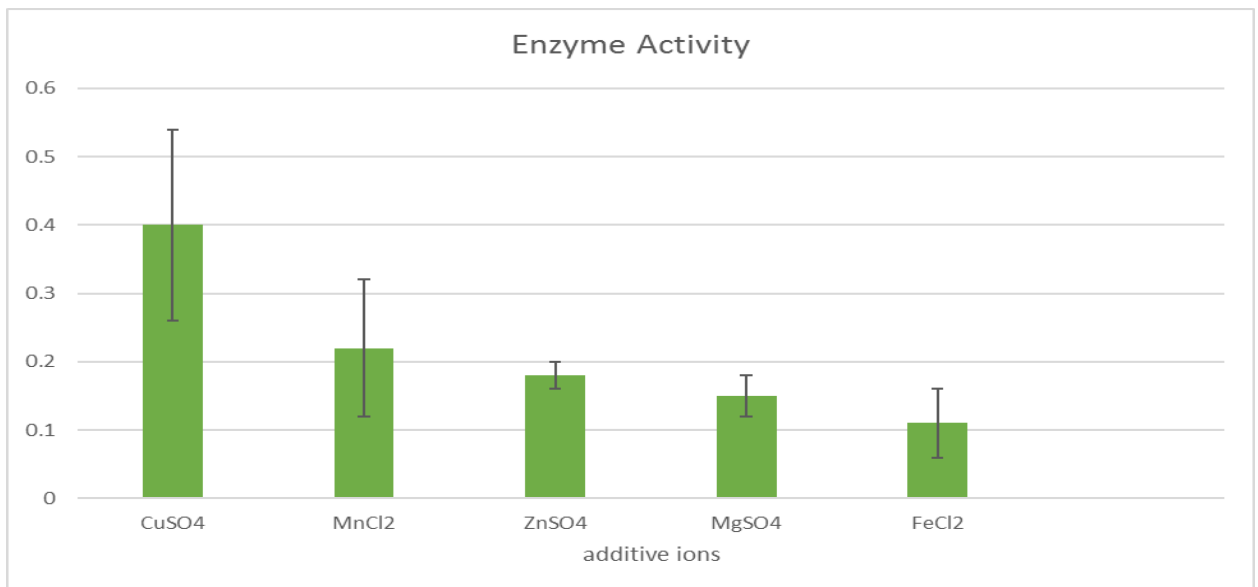


Figure 6. Effect of different metal ions on uricase activity

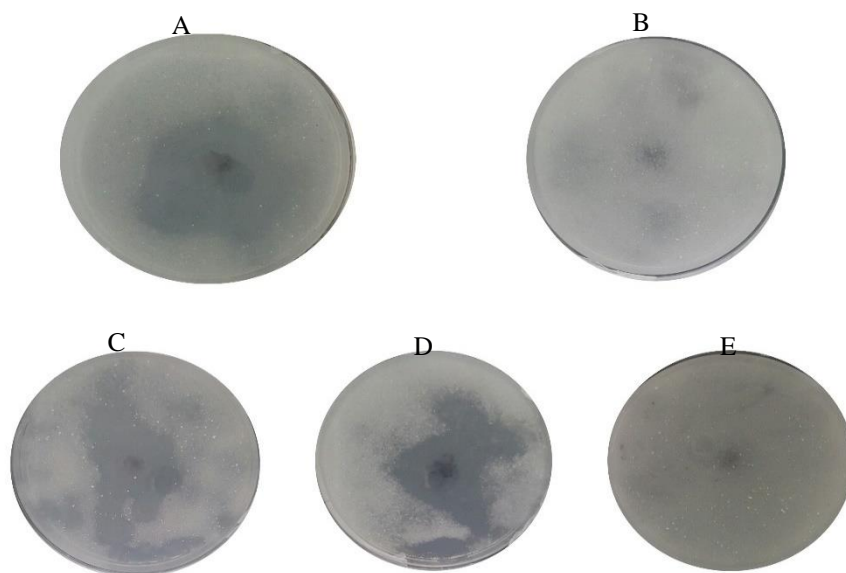


Figure 7. *Aspergillus niger* on uric acid plates with different additive ions. A) Cu^{2+} B) Mn^{2+} C) Zn^{2+} D) Mg^{2+} E) Fe^{2+}

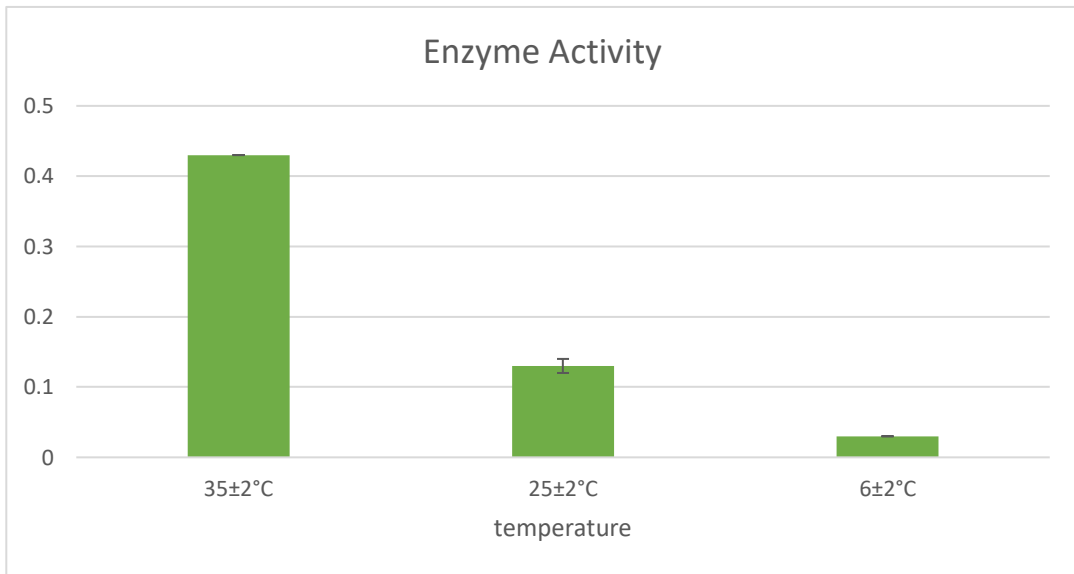


Figure 8. Effect of temperature on uricase activity

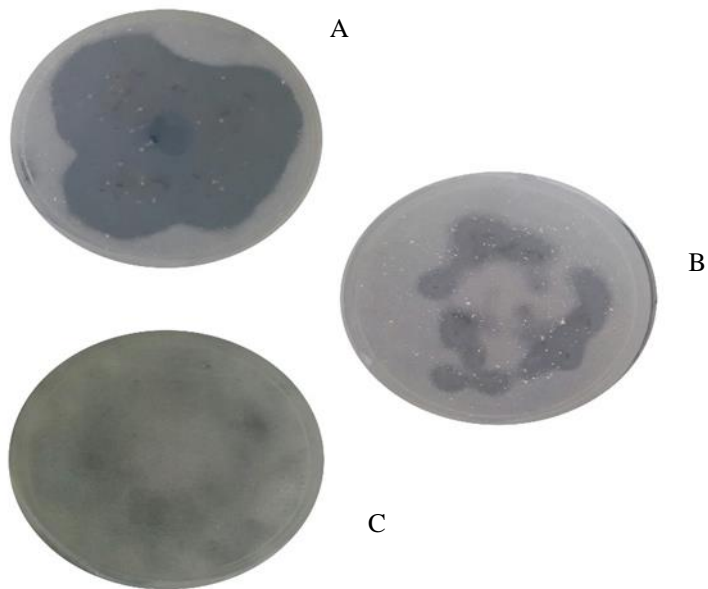


Figure 9. *Aspergillus niger* on uric acid plates at different temperatures. A) 35±2°C B) 25±2°C C) 6±2°C

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