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# Isolation of Novel *Alcaligenes faecalis* for Production of Medically Useful Enzyme Uricase

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**Abstract:** Uricase (EC 1.7.3.4) enzyme is widely used for uric acid detection in clinical analysis and there are many reports on uricase enzymes for efficient treatment of gout and hyperuricemia. This study was conducted in order to investigate new sources of uricase hyper producer. Uricase producing bacteria were successfully isolated from poultry farm soil samples on the basis of their uricolytic activity on uric acid containing agar medium. The isolated bacterium was identified as *Alcaligenes faecalis* on the basis of 16S rRNA sequence and phylogenetic tree analysis. This is the first report of production of clinically important enzyme uricase from isolated *Alcaligenes faecalis*. The uricase was present as extracellular enzyme. The extracellular uricase activity of 29.2±0.92 IU was observed, when the isolate was cultured at 30°C and pH 6.5 for 42 h on nutrient broth medium containing 0.4 (%, w/v) uric acid .

**Keywords:** *Arlcaligenes faecalis*, isolation, 16S rRNA, uric acid, uricase, Hyperuricemia, Gout.

## Introduction

Therapeutically important enzyme uricase (EC 1.7.3.3, UC) belongs to the class of the oxidoreductases (**Fig. 1).** Uricase catalyzes the opening of the purine ring of uric acid through oxidative process to form allantoin, having 5-10 times more solubility than uric acid [15]. Uricase is produced as a truncated, 10 amino acids long, inactive protein fragment in humans and apes because of insertion of non sense codon into uricase producing gene [7]. The biological reason is still not clear for the extinction of uricase activity in humans and certain primates. According to one view, uric acid being powerful antioxidant this loss has a distinctly beneficial effect due to scavenging of free radicals by it. Therefore, these properties of uric acid have contributed to lowering of the cancer rate and increase in hominoid life span [17].The plasma concentration of uric acid in humans is high, due to absence of uricase. This uric acid accumulates in form of mono sodium urate crystals and causes the damage to liver and kidney [12, 22].

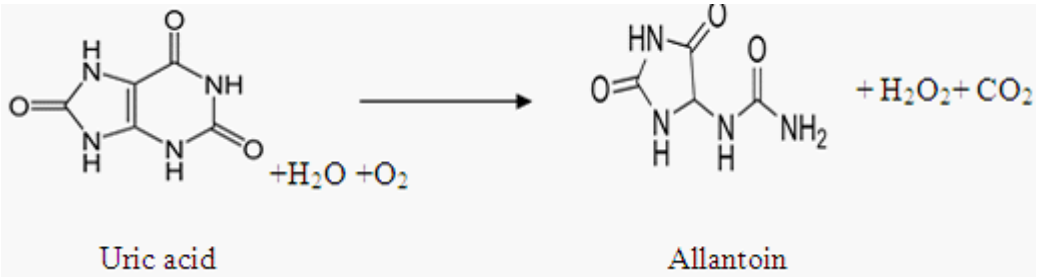


**Fig.1.** Reaction catalyzed by uricase

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The normal serum urate level of uric acid is between 2.18-7.7 mg/dL. The abnormal rise of uric acid can lead to the development of disease known as gout and hyperuricemia. Condition of gout is a characterized by joint and kidney inflammation caused by high levels of uric acid in the bloodstream and tissues. Other diseases associated with hyperuricemia such as type 2 diabetes which causes insulin absorption resistance in tissues, increase of nephropathy progression, and tumor lysis syndrome [6, 19].

The treatment of hyperuricemia has been performed with drugs such as allopurinol that decrease amount of uric acid synthesis by inhibiting xanthine oxidase enzyme. However, these treatments can sometimes prove to be inefficient, leading to serious clinical complications [9, 29]. Administration of uricase has proved to be a good alternative to treat gout. Uricase is useful for enzymatic determination of uric acid in biological fluids for clinical analysis [11]. Immobilized uricase has also been used as a uric acid biosensorand additive in commercial formulations of hair coloring agents [13, 25]. Rasburicase (Fasturtec/Elitek) is a market name given to the *Aspergillus flavus* uricase. The gene for the uricase isolated from *Aspergillus flavus* has been expressed in *Saccharomyces cerevisiae*. This uricase has been approved for clinical use by FDA [14]. Studies showed that Rasburicase has been more effective than other drugs in the treatment of hyperuricemia due to low incidence of hyper sensibility reaction [27, 33]. Native enzyme may be obtained from several microorganisms of the genus *Micrococcus*, *Brevibacterium*, *Streptomyces*, *Candida*, *Bacillus, Pseudomonas, Arthrobacter*, and *Aspergillus* [16, 18, 20, 31]. Uricase from thermophilic *Bacillus* sp. TB-90 and *Bacillus firmus* DWD-33 has been extensively studied for diagnostic purposes due to high activity and thermostability at wide range of pHs [21, 34].

The production of recombinant strain carrying uricase genes from *Pseudomonas aeruginosa* Ps43 and *Candida utilis* has also been reported using *E. coli* and *Hansenula polymorpha*, respectively [10, 30]. Although uricase has been produced from numerous sources but its rising importance in treatment and diagnosis of hyperuricemic diseases, leads to the screening of new sources with unique properties for economical production of this enzyme to increase its usefulness. The present study focus on the isolation of a novel *Alcaligenes faecalis* with extra cellular uricase activity.

## Material and method

### *Isolation, screening and selection of bacteria*

Soil samples from various poultry farms located at different regions of Himachal Pradesh, India were collected and used for the isolation of extracellular uricase producing bacteria. Isolation was carried on nutrient agar media with 0.3 % uric acid concentration. The initial pH was adjusted to 7.0 with 0.1 N NaOH. 1gm soil sample was added to 10 ml of sterile physiological saline (NaCl 0.89 %, w/v) and then serially diluted up to $10^{-8}$ dilutions. From the different dilutions, 0.1 mL samples were placed on nutrient agar plates and spreaded. Plates were incubated at 30$^{o}$C and analyzed up to 36 h. Microorganisms were selected on the basis of clear zone around the colony on the agar plates containing uric acid.

### Enzyme assay

The enzymatic assay for detecting uricase activity was carried out by the method as described by Mahler *et al* [24]. Reaction mixture consists of 3 mL of 20 mM boric acid buffer of pH 8.5, 75 µL of 3.57 mM uric acid solution and 25 µL of enzyme. To equate the volumes of reaction mixture, 25 µL of buffer was added in blank instead of the enzyme solution. The blank and the test solutions were incubated at 30°C for 20 minutes. The decrease in the uric acid concentration in test solution due to the uricase action was measured with the aid of a UV-visible spectrophotometer at 293 nm. One unit of enzyme activity was defined as the amount of uricase required to convert 1 mmol of uric acid into allantoin per minute at 30°C and at pH 8.5, considering milli molar extinction co-efficient of uric acid at 293 nm as 12.6 $mM^{-1}$ $cm^{-1}$.

### Identification of microbial strain on the basis of 16Sr RNA sequencing

### *DNA Preparation and PCR Amplification*

DNA was extracted from the isolated uricase

producing bacterial strain. Its quality was evaluated on 1.2 % Agarose Gel. Fragment of 16S rDNA gene was amplified with the help of PCR from the above isolated DNA. A single discrete band of 1500 bpPCR amplicon then resolved on 1 % agarose Gel.

The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was done with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730 xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

**16S rRNA sequencing and data analysis**

The 16S rDNA gene sequence was used to carry out BLAST with the nr database (non reductant database) of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4. The evolutionary history was inferred using the Neighbor-Joining method [28].

**The physicochemical analysis of uricase production conditions for by *A. faecalis.***

***Selection of medium for production of extracellular uricase from A. faecalis***

The medium producing better growth and high uricase activity was needed to be selected and optimized. Various media (M-1 to M-9) which have been earlier used for the production of uricase were studied in order to find suitable medium. The initial pH of the seed medium was set at 7.0. The 0.4 %, (v/v) of the seed culture were used to inoculate the production medium. The inoculated production medium was incubated at 30°C for 36 h at 150 rpm. Thereafter, the cell supernatant was harvested and was used for enzyme assay.

**The effect of inducer (uric acid) on the production of extracellular uricase by *A. faecalis***

The effect of inducer on production of uricase was studied with or without the addition of (0.3 %, w/v) uric acid in seed and production medium and its effect on growth and uricase production was studied.

**Effect of inducer concentration on the production of uricase**

The seed medium and production medium supplemented with different concentrations (0.1 to 0.6 %, w/v, respectively) of uric acid was inoculated with *A. faecalis* cells. The inoculated flasks were incubated at 30°C for 36 h at 150 rpm in order to evaluate the optimum uric acid concentration in the seed and production medium. After incubation the culture medium was centrifuged at 10,000 rpm for 10 min at 4°C and uricase activity was measured under standard assay conditions.

**Effect of the different physiological conditions on the production of uricase by *A. faecalis***

Various physiological conditions such as pH (5.0 to 8.0), temperature (25 to 45°C), and agitation rate (100 to 200 rpm) were studied in order to obtain most suitable conditions for production of uricase. The composition of both seed and production media was (%, w/v): dextrose-1.0; yeast extract-0.2; NaCl-0.5 and uric acid-0.4.

**Course of cultivation of *A. faecalis***

The production medium (pH 6.5) containing (%, w/v) dextrose-1, yeast extract-0.2, NaCl-0.5 and uric acid 0.4, was inoculated with 4 %, v/v seed of 24 h age and flasks were incubated at 30°C (150 rpm). Samples were withdrawn at regular time intervals of 12 h from production and were analyzed for cell growth, final pH and uricase activity up to 72 h.

**Results and discussion**

***Isolation and selection of bacteria***

Appropriately diluted poultry farm soil samples were spreaded on uric acid containing agar plates. The bacterial strains were isolated on the basis of their uricolytic activity on nutrient agar medium containing uric acid (Fig. 2). The selected bacterial isolates were grown in nutrient broth contain 0.3 % (w/v) uric acid and their extracellular uricase activity was checked after 36 h of incuba-

**Fig. 2.** Bacterial strain USP-2 showing zone of clearance after 36 h

tion. The isolate USP-2 was selected as the most potential strain, having uricase activity of 12.6 ± 0.82 IU. The isolate was then identified on the basis of biochemical tests followed by 16SrRNA sequencing.

**Identification of bacterial isolate USP-2**

Morphologicallythe isolate was found to be Gram negative rod and it forms creamish yellow colony with rough margin. This isolate was catalase, citrate and oxidase positive and indole negative. It also reduces glucose, mannitol and maltose into corresponding acids.Sequence providing information about the close homologs for the bacterial isolate USP-2 has been given in (Table 1.) Based on nucleotide homology and phylogenetic analysis (Fig. 3), isolate, USP-2 was found similar to *Alcaligenes faecalis* strain ZJB-09133 (GenBank Accession Number: GQ438851.1)

**The physicochemical analysis of production conditions for maximum uricase production from *A. faecalis***

The various experiments were carried out in Erlenmeyer flasks (250 mL) to obtain best physicochemical conditions for production of uricase by *A. faecalis*. The flasks were incubated in temperature controlled orbital shaker and periodical sampling was carried out for the determination of cell growth (mg/mL), final pH and uricase activity (IU).

**Selection of medium for production of extracellular uricase by *A. faecalis***

The culture of *A. faecalis* was grown on nine different media, reported for the production of extracellular uricase (Table 2). Among the nine media tested, the maximum growth (3.5±0.17 mg/mL)and activity of uricase (19.4±1.61 IU)was

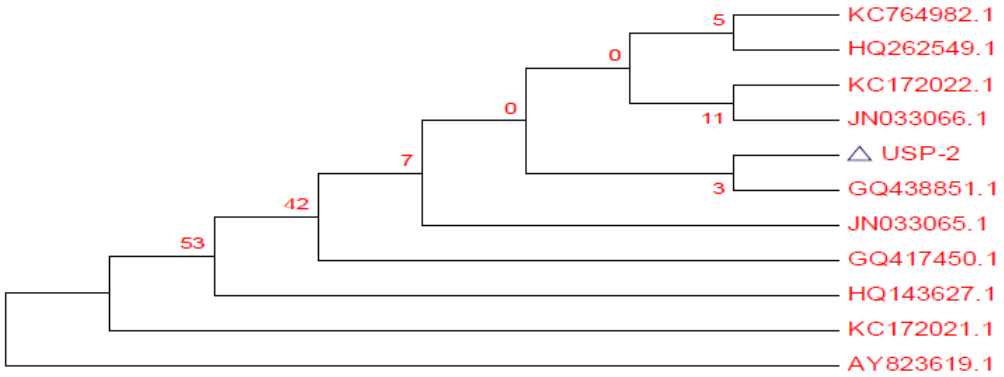


**Fig. 3.** Phylogenetic tree of USP-2 isolate. Phylogenetic trees wereconstructed by the neighbor-joining method using MEGA software, version 5.2.

**Table 1. Sequence producing significant alignments**

| Accession no. | Description | Max score | Total score | Query coverage % | E value | Max ident % |
|---|---|---|---|---|---|---|
| KC764982.1 | *Alcaligenes faecalis* strain T196 | 2597 | 2597 | 100 | 0.0 | 100 |
| HQ262549.1 | *Alcaligenes* sp. S3 | 2597 | 2597 | 100 | 0.0 | 100 |
| KC172022.1 | *Alcaligenes faecalis* strain SH15 | 2595 | 2595 | 99 | 0.0 | 100 |
| KC172021.1 | *Alcaligenes faecalis* strain SH14 | 2591 | 2591 | 99 | 0.0 | 100 |
| JN033066.1 | Uncultured bacterium clone 3-42028 | 2591 | 2591 | 99 | 0.0 | 99 |
| JN033065.1 | Uncultured bacterium clone 3-42007 | 2591 | 2591 | 100 | 0.0 | 99 |
| HQ143627.1 | *Alcaligenes faecalis* strain TZQ4 | 2591 | 2591 | 100 | 0.0 | 99 |
| GQ438851.1 | *Alcaligenes faecalis* strain ZJB-09133 | 2591 | 2591 | 100 | 0.0 | 99 |
| GQ417450.1 | Uncultured *Alcaligenes* sp. clone F2 aug.18 | 2591 | 2591 | 100 | 0.0 | 99 |
| AY823619.1 | *Alcaligenes faecalis* strain MT1 | 2591 | 2591 | 100 | 0.0 | 99 |

**Table 2. List of media for the production of uricase**

| Medium no. | Composition (g/L) | Uricase activity (IU) | Cell growth (mg/mL) |
|---|---|---|---|
| M-1 | Tryptone-5.0, soya extract-10.0, dextrose-2.0 uric acid -3.0 | 13.9±1.10 | 3.02±0.16 |
| M-2 | Sucrose -150.0, yeast extract-20.0, uric acid-3.0 | 4.3±0.7 | 1.80±0.07 |
| M-3 | Dextrose -10.0,Yeast extract - 2.0, NaCl -5.0, Uric acid - 3.0 | 19.4±1.6 | 3.21±0.17 |
| M-4 | Sucrose - 20.0, $KH_2PO_4$ - 1.0, $MgSO_4$ - 0.5, Uric acid -1.0 | 4.9±0.7 | 2.12±0.20 |
| M-5 | Peptone -10.0, Yeast extract - 10.0, NaCl -5.0, Uric acid -3.0 | 8.9±1.6 | 2.41±0.09 |
| M-6 | Dextrose - 20.0, Peptone - 30.0, $MgSO_4$ - 0.5, $KH_2PO_4$ 1.0 Uric acid-0.3 | 16.8±0.9 | 3.16±0.07 |
| M-7 | Luria broth - 23.0, Uric acid - 3.0 | 10.9±1.8 | 2.82±0.09 |
| M-8 | Nutrient broth -13.0, Uric acid-3.0 | 12.7±1.2 | 2.40±0.06 |
| M-9 | Peptone-20.0, Sucrose-30.0, $KH_2PO_4$- 1.0, $MgSO_4$ - 0.5, Uric acid-0.3 | 2.8±0.5 | 1.60±0.11 |

obtained in medium 3 containing (%, w/v) dextrose-1, yeast extract-0.2, NaCl-0.5 and uric acid 0.3**.** However, lowest uricase activity (2.8±0.5 IU) was detected in medium 9. Similar media was selected for the production of uricase from *P. aeruginosa*[4]. Uricase production has also been

studied from *Gliomastix gueg* by Atalla and colleagues[5] and it was found that uric acid medium containing (g/L): uric acid 1.0; $K_2HPO_4$1.0; $MgSO_4$0.5; NaCl 0.5; $FeSO_4$0.01 and sucrose 20.0 was best suited for the uricase production by *G. gueg*. Another study on the production of uricase by *Gliocladium viride* showed that medium containing (g/L): sucrose 20.0; uric acid 2.0; $K_2HPO_4$1.0; $MgSO_4$.7 $H_2O$ 0.5; NaCl 0.5 and $FeSO_4$0.01 was most suitable for enzyme production [26].

**The effect of inducer (uric acid) on the production of extracellular uricase by *A. faecalis***

Inducer plays an important role in production of uricase and enhancement of the growth. The role of inducer (uric acid 3 %, w/v) in seed and production medium for the production of uricase by *A. faecalis* was studied in four different combination of seed and production medium (Table 3). Seed and production medium supplemented with uric acid was proved to be best combination for growth (3.2±0.24 mg/mL) and uricase activity (20.7±1.07 IU). These results clearly indicate that uricase from *A. faecalis* is an inducible enzyme and presence of uric acid in both seed as well as production medium is essential for maximum growth and enzyme production.

**Effect of inducer concentration on the production of extracellular uricase by *A. faecalis***

The addition of uric acid in production medium plays an important role for production of uricase. It has been reported that uric acid is required as inducer in production medium for uricase synthesis[2]. The uricase production by *A. faecalis* was studied by preparing the media (pH 6.5) with varying concentration of uric acid incubated at 30°C for 36 h (150 rpm). Maximum growth and production of uricase enzyme(23.2±0.91 IU) was observed at 0.4 (%, w/v) uric acid concentration in production medium (Fig. 4).

**Effect of the different physiological conditions on the production of extracellular uricase by *A. faecalis***

The variation of different physiological conditions greatly affects production of uricase. In order to find out optimum level, various physiological conditions such as pH (5.0 to 8.0), temperature (25 to 45°C), and agitation rate (100 to 200 rpm) varied and their effect were studied. The optimum level of pH, temperature and agitation for maximum production of uricase were found at pH 6.5, temperature 30°C and agitation rate 150 rpm, respectively (Fig. 5 a, b, c).

The maximum uricase activity (26.6±0.89 IU) was observed after optimization of physiological conditions. The present study was slightly in agreement with those of Tohamy and Shindia [32] and Yazdi *et al* [35] who found that pH 6.0 was optimum for uricase production from *A. flavus* and *Mucor hiemalis*, respectively. In various studies, the optimum temperature for uricase production has also been reported to be 30°C [3, 8, 32, 35]. The results of present work coincided with the study carried out by Atalla *et al*[5] on *G.gueg*, having optimum agitation rate of 150 rpm for uricase production.

**Course of cultivation of *A. faecalis***

The production medium (pH 6.5) containing (%,

**Table 3. Effect of inducer on uricase production by *A. faecalis***

| No. | Inducer | Cell growth (mg/mL) | Uricase activity (IU) |
|---|---|---|---|
| 1 | $S^+P^+$ | 3.2±0.24 | 20.7±1.07 |
| 2 | $S^-S^+$ | 3.1±0.28 | 15.7±1.10 |
| 3 | $S^+P^-$ | 2.7±0.26 | 12.5±0.94 |
| 4 | $S^-P$ | 2.9±0.24 | 0.0 |

[#]'S'-seed medium; 'P'-production medium;

'+' and '-' indicates medium supplemented with or without uric acid

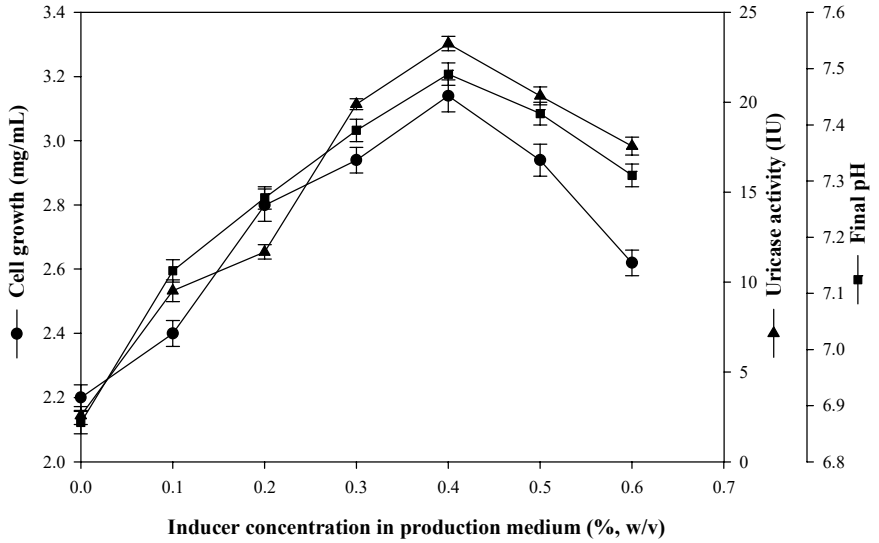


**Fig. 4.** Effect of concentration of inducer on uricase production by *A. faecalis*

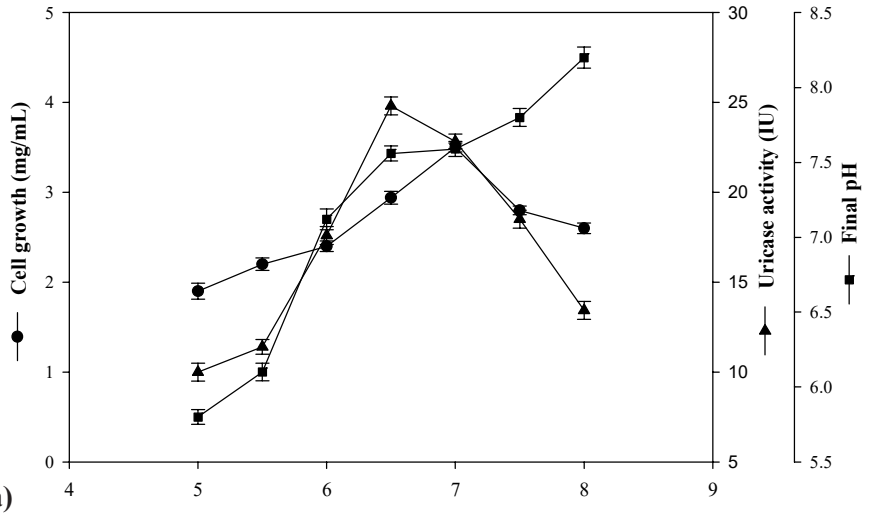


**Fig (a)**

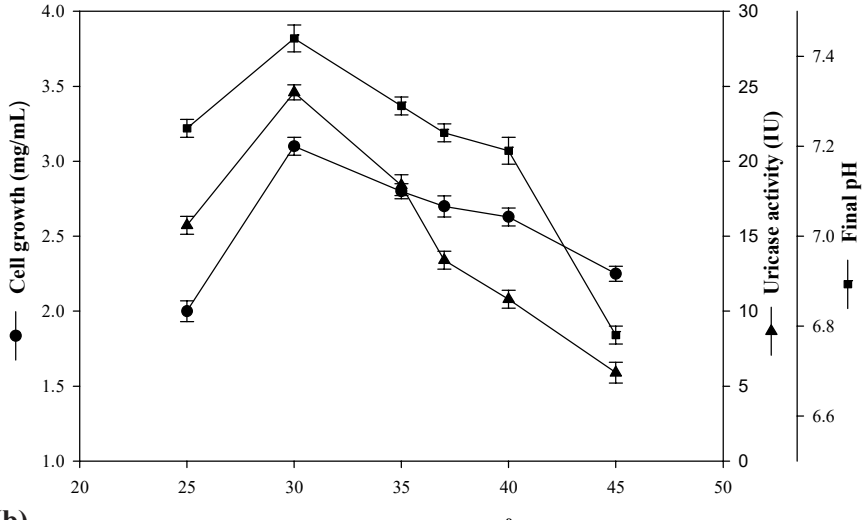


**Fig (b)**

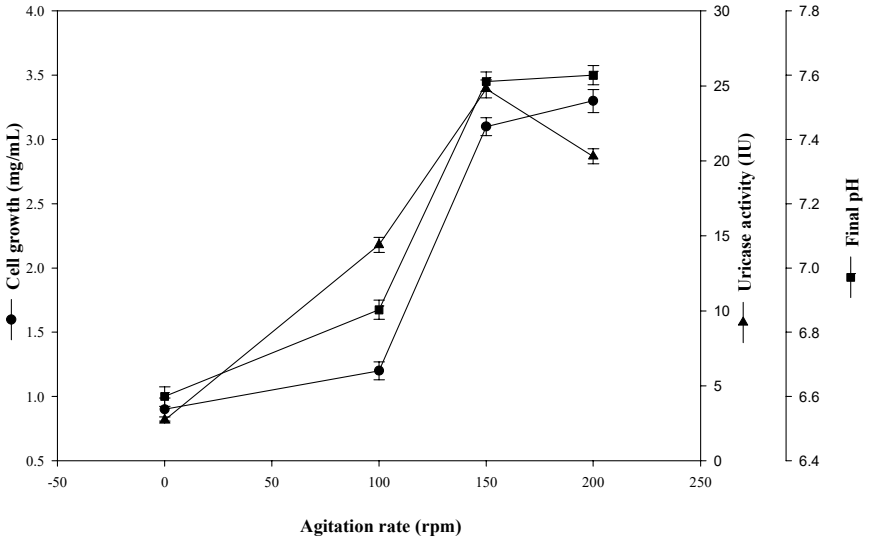


**Fig (c)**

**Fig. 5.** Effect of temperature (a), pH (b) and agitation rate (c) on uricase production by isolated *A. faecalis*

w/v) dextrose-1, yeast extract-0.2, NaCl-0.5 and uric acid 0.4, was inoculated with 4 %, v/v seed of 24 h age and flasks were incubated at 30°C (150 rpm). During course of cultivation of *A. faecalis* thegrowth and culture pH was found to increase up to 60 h. The increase in pH might be due to utilization of uric acid by culture. The maximum growth (3.46±0.19 mg/mL) was observed at 60 h and thereafter that cell mass decreased. The maximum uricase activity (29.2±0.92 IU) was observed at 42 h of incubation (Fig. 6). The present result was slightly in agreement with work carried out by Anderson and Kumar on *P. aeruginosa* [4]. They have optimized the 36 h of incubation time for the maximum production of uricase. Atalla *et al.* [5] have found that 8 d of incubation is most suitable for uricase production by *G. gueg*. Abd El Fattah and Abo Hamed have produced uricase by *A. flavus* and *A.terreus* after 4 d incubation and by *Trichoderma* sp. after 6 dof cultivation [1].

## Conclusion

The immunogenic reaction associated with the

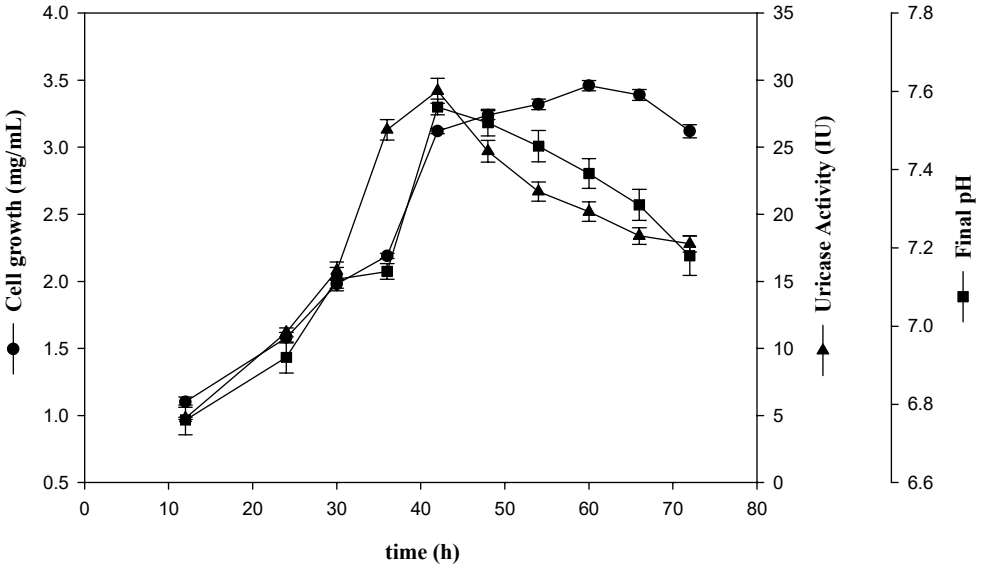


**Fig. 6.** Course of cultivation of *A. faecalis*

currently used clinical preparation of uricase has necessitated the search for the alternative sources. The uricase from different sources show no immunological cross reactivity and so can provide an alternative therapy for a patient who has become hypersensitive to the enzyme preparation of one source. Hence, there is need to continue the examination of microbe to find newer isolates which could prove to be ideal sources of this therapeutically important enzyme. The focus of the present study was on the isolation of new microbe which can produce extracellular uricase. The isolate *Alcaligenes faecalis* has not so far been reported for the production of uricase. Preliminary physicochemical analysis for production of uricase by *Alcaligenes faecalis* has resulted into two fold enhancement in the enzyme production.

**Acknowledgment**

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