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Continuous production of Urocanic acid by immobilized *Pseudomonas aeruginosa* species

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ABSTRACT: Urocanic acid (UA), an intermediate of L-histidine catabolism is commonly used as sun-screening agent in cosmetics and medicines. In the present work, an effort has been made to isolate the urocanic acid producing strain (Pseudomonas aeruginosa) and optimized at varied culture conditions in submerged fermentation like pH, temperature, incubation period and substrate concentration. Effect of carbon source, nitrogen source and metal ions were also studied. The results revealed that maximum Urocanic acid production was attained at pH 7.5 and temperature 40 ^{0}C using 0.4 % of histidine after 96 h of incubation. Carbon source (Glucose 2%, 2.104 mg/ml), nitrogen source (yeast extract 0.5 %, 1.936 mg/ml and metal ions (Mg^{++} 0.1mM, 1.523 mg/ml) further enhances the Urocanic acid production. Immobilization of Pseudomonas aeruginosa in 1.5 % of calcium alginate beads increased the yield up to 2.245 mg/ml. Moreover, repetitive batch operation and storage stability study of immobilized cells revealed maximum UA production up to five batches of operation. When stored at 4 ^{0}C, the immobilized whole cells remained stable up to 4 weeks. Hence immobilized whole cells of Pseudomonas aeruginosa were beneficial for industrial urocanic acid production.

Key words: Pseudomonas aeruginosa / Urocanic acid/Optimization / Immobilization.

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I. INTRODUCTION

L-histidine ammonia-lyase [E.C.4.3.1.3] an enzyme responsible for conversion of L-histidine to ammonia and glutamic acid through urocanic acid (UA) have been described well from a variety of microorganisms viz. *Pseudomonas putida, Aerobacter aerogenes, Bacillus subtilis, Bacillus cereus, Mycobacterium avium, Salmonella typhimurium, Serratia marcescens, Vibrio cholera, Pseudomonas fluorescens, Pseudomonas aeruginosa* and *Pseudomonas testosteroni* [1]. The enzyme activity also occurs in liver, skin, blood serum and stratum corneum of higher animals and in plants such as spinach and sunflowers [2,3]. Production of UA may be suggested as a useful alternative to histamine as a spoilage index in *Scombroid* and other fish that are rich in endogenous histidine [4]. Metabolites of histidine (UA) produced in animal tissues functions as protective agent against ultraviolet radiation [5]. Commercially UA is used as sun-screening agent in cosmetics and medicines. The production of UA is usually carried out by microbial and enzymatic conversion using L-histidine [3,7]. Optimization and genetic modification studies was carried out for *Serratia marcescens* for UA production respectively [8].

One of the major advances in optimizing microbial biotechnological process lies in immobilization technology which can be defined as any process that restricts substrate or cells inside a given structure and limits their free diffusion or movement. Moreover, immobilization offers advantages such as ease of handling, easy cell separation from liquid medium, the possibility of repeated and continuous use of biocatalysts as well as an increase in enzyme stability and longitivity in contrast to that of free cells. These factors lead to a subsequent increase in bioprocess efficiency [9,10] .

Literature survey revealed efficient UA production by immobilization methods [11,12] The isolation and characterization of new producer strains using carbon and nitrogen sources appears to be also essential for the improvement of process economy [13]. Taking these points under consideration, an effort has been made to isolate the *Pseudomonas aeruginosa* strain from garden soil sample, optimized at varied culture conditions and immobilized in calcium alginate beads. These immobilized whole cells were used efficiently for industrial UA production.

II. MATERIALS AND METHODS

Bacterial isolation and culture conditions

Urocanic acid producing microorganisms were isolated from garden soil, NES Science College, Nanded, by spread plate method using successively enriched broth containing 0.1-0.6 % histidine in peptone water, pH 7.0, at 30 ^{0}C for 24-48 h. Routine identification and quantitative estimation of the UA and amino acids formed were made by paper chromatography using n-butanol: acetic acid: water (4:1:1). After development, the chromatogram was spread with Pauly reagent or 0.2% ninhydrin in 80 % ethanol. Ammonia was estimated by Nesslers reagent. *Pseudomonas aeruginosa*, a producer strain was identified based on cell morphology, cultural and biochemical characteristics and maintained on nutrient agar throughout the experimental work at 4 ^{0}C temperature. Further confirmation was done by 16S rRNA gene sequencing experiment. For inoculum preparation the culture was reactivated by transferring into fresh broth medium containing 0.2 % histidine as inducer and incubated at 30 ^{0}C for 24 h. Previously UA was produced by surface and submerged level in 100 ml of the histidine broth (0.2 %) at 30 ^{0}C for 192 h on shaker. During fermentation the physiological parameters like pH, growth, enzyme activity, histidine degradation was UA production was checked to obtain maximum enzyme and UA production (Fig 1).

Methods of analysis

L-histidine ammonia lyase activity was carried out by spectrophotometeric measurements of UA formed from L-histidine. The cells were harvested by centrifugation from 2 ml of the cultured broth, washed twice with saline and suspended in reaction mixture to a final volume of 4 ml. The reaction mixture contained 0.15 M L-histidine hydrochloride monohydrate adjusted to pH 9.0 with 5 N NaOH, and was incubated for 1 h at 37 ^{0}C. After appropriate dilution with 0.01 M potassium phosphate (pH 7.4), the amount of urocanic acid formed was assayed by measuring the optical density at 277 nm. Amount of urocanic acid was estimated from standard graph. One unit of L-histidine ammonium lyase activity was defined as that activity which converted 1 µmol of L-histidine to urocanic acid per min under the conditions of the assay. Total enzyme activity, i.e. amount of formed enzyme, was expressed in terms of units per milliliter. The assay of histidine was carried out by Paulys method. Ammonia was assayed by the nesslerization method. For the estimation of growth, optical density was measured at 610 nm and by dry cell weight (mg/ml) (UV-Visible Schimadzu spectrophotometer). pH was measured by pH meter (Bangalore genei).

Optimization of cultural conditions for Urocanic acid production

The cultural conditions like incubation period (24h–144h), pH (6.0-9.0), temperature (30 ^{0}C- 55 ^{0}C) and histidine concentration (0.1-0.6 %) were optimized for maximum UA production. Effect of different 2% carbon sources (glucose, sucrose, lactose, maltose, mannitol), 0.5 % nitrogen sources (ammonium sulfate, urea, yeast extract, beef extract) and metal ions (K^+, Fe^{++}, Cu^{++}, Zn^{++}, Mg^{++}) on UA production was carried out in 250 ml of Erlenmeyer flask containing 100 ml of histidine broth (0.2%). All the flasks were inoculated with 2 % v/v of inoculum and incubated in rotary shaker at 140 rpm for 96 h. Fermentations were performed in triplicate culture flasks and analysis was carried out in triplicate. The data given here are the average of three measurements.

Extraction of Urocanic acid

UA was extracted from fermentation broth by simple method. The broth was centrifuged to remove the cells, heated and acidified with hydrochloric acid up to pH 4.8. The whole content was chilled immediately to 0^{0}C to get precipitated UA crystals. The reaction mixture was filtered and recrystallized from hot water to yield pine leaf like crystals of UA. Purity of the compound was checked microscopically, by TLC and by Melting point.

Immobilization of Pseudomonas aeruginosa strains for UA production in sodium alginate gel

Sodium alginate (1, 1.5 and 3% w/v) was dissolved in deionized water and autoclaved at 121 ^{0}C for 15 min respectively. After cooling to 40 ^{0}C the alginate slurry was mixed with bacterial cell suspension (2 % v/v, 2 x 10^6 bacterial cells/ml) and stirred to obtain homogeneous mixture. The mixture was transferred into a sterile syringe (10 ml) and the drops were extruded into a solution of 0.5 M $CaCl_2$ (250 ml) in a beaker from 5 cm height to obtain beads of uniform size. The beads were kept for curing at 4 ^{0}C for 1 h. The cured beads were washed 3-4 times with sterile distilled water to remove unbound bacterial cells. All operations were carried out aseptically in laminar flow (Remi). The beads were then stored at 4 ^{0}C until further use.

Determination of immobilized whole cell enzyme activity and UA production

The alginate beads were weighed after gentle drying on filter paper and then placed in a sterile flask. The reaction mixtures containing 20 g of L-histidine hydrochloride monohydrate and 0.2 ml of 50 % aq. solution of triethanolamine lauryl sulfate (TEALS), to final volume of 200 ml. The reaction mixture was adjusted to pH 9 with 5N of NAOH and incubated at 40 ^{0}C for 12 h. UA production increases with consumptions of L-histidine and estimated by above discussed method.

Repetitive batch operation

Operational stability of immobilized whole cells was examined by repetitive batch bioconversion. Immobilized whole cells were packed in a column (1 x 25 cm). L-histidine solution (0.25mM) was circulated between this packed bed column and the product was allowed to flow at the rate of 0.5 ml/min for 6 h (one reaction cycle). At the end of each reaction cycle the column was flushed with potassium phosphate buffer (pH 7.4, 15 min).At the end of each reaction cycle, the reaction mixture was collected and processed for recovery of UA, as per method described above. Each of the assays was carried out up to 5 cycles.

Storage stability of immobilized cells

The immobilized cells were stored in 2% w/v of $CaCl_2$ solution at 4 ^{0}C for 8 weeks to investigate their storage stability.

III. RESULTS AND DISCUSSION

Isolation, Screening and time course study for Urocanic acid production

Histidine degrading bacterial cells were isolated from a successive enriched growth medium and screened for their enzyme activity and UA production. Out of 26 different bacterial species, the isolated strain showed maximum UA production, determined by chromatogram and deep orange colorization with Nesselers reagent. The isolate was identified primarily by cell morphology, culture and biochemical characterization and finally by 16S rRNA gene sequencing. Based on these results the organism was identified and confirmed as *Pseudomonas aeruginosa* strain. The time course of L-histidine degradation and growth in submerged and surface culture conditions was assessed using the isolated strain as per Fig 1. During degradation a continuous rise in pH, growth, enzyme activity, histidine degradation and UA production was achieved in both types of fermentations. L-histidine (25%) concentration was remained in the fermentation after 144 h of incubation with maximum UA production 1.02 mg/ml having pH 8.5. Negligible amount of L-glutamic acid production as final product was obtained late in the fermentation. This may be due to UA degradation. Similar results were observed in *A. liquidum* strain submerged fermentation for maximum L-histidine ammonia-lyase formation [3].

Optimization of culture conditions for UA production

To establish the most advantageous cultural conditions for the UA production, effect of various parameters viz. L-histidine concentration (0.1-0.6%), incubation period (24-144 h), pH (6-9) and temperature (30-55^{0}C) were investigated with *Pseudomonas aeruginosa* strain.

Histidine was used as sole source of carbon and nitrogen for UA production by *Pseudomonas aeruginosa* stain. It showed highest UA production at 0.4% (1.25 mg/ml) with enzyme activity (0.0468 U/ml) when compared with 0.3 % (0.92 mg/ml, 0.0517 U/ml) after 96 h of incubation. The decrease in enzyme activity at that instant may be due to saturation of the enzyme active sites with the substrate, while increase in UA production is related to its active conversion of L-histidine to UA as observed with 0.4 % of histidine concentration (Table 1). In enteric bacteria the histidase formation was reported to be controlled by induction and catabolite repression, where L-histidine or UA act as inducer for histidase, therefore the substrate concentration effect was studied [8].

The effect of incubation period on UA production was determined up to 144 h, where the highest UA is produced (1.23 mg/ml, 0.052 U/ml) after 96 h of incubation. The further decrease in enzyme activity and UA may relate with its urocanase activity (Table 2). Urocanase activity determination was carried out and found to be present after 120 h of incubation with little amount of glutamate in broth detected by chromatography technique.

Effect of pH and temperatures was also carried out as it affect the enzyme production in fermentation medium .Any change in these parameters induces the morphological changes in microbes and in enzyme secretion [13]. The maximum UA production was found at pH 7.5 and temperature 40 ^{0}C as per Table 3 and 4 respectively. These results coincide with the L-histidine ammonia-lyase activity of *A. liquidum* strain (pH 9, temperature 40 ^{0}C).

Previous study on catabolite repression of histidase formation was carried out in *S. marcescens*, using different carbon sources. Therefore we examined the effect of sugars viz. glucose, sucrose, maltose, lactose, mannitol on UA production [8]. Moreover, effect of different carbon sources is also essential for the improvement of process economy. The results revealed that lactose strongly repressed histidase formation (0.043 U/ml, 1.482 mg/ml) as compared to other sugars, glucose (0.123 U/ml, 2.104 mg/ml), sucrose (0.094 U/ml, 1.923 mg/ml), mannitol (0.098 U/ml, 1.889 mg/ml) and maltose (0.072 U/ml, 1.742) after 96 h of incubation. In case of *A. liquidum* UA production study, maximum enzymatic conversion was obtained with 2% glucose (Table 5).

The external nitrogen source effect revealed that maximum UA production was obtained using yeast extract (1.939 mg/ml, 0.096 U/ml) followed by beef extract (1.729 mg/ml, 0.07 U/ml), urea (1.692 mg/ml, 0.065

U/ml) and ammonium sulfate (1.462 mg/ml, 0.039 U/ml). A decrease in histidase enzyme activity relate with ammonium sulfate was reported in *S. marcescens* mutant [7] (Table 6).

The other important component is metal ions that act as cofactor for enzyme and stimulate its production. Enzyme activity was increased up to 0.052 U/ml using Mg^{++} (1.523 mg/ml) .While other metal ions Cu^{++} and K^{+} also found to be effective for UA production. Zn^{++} and Fe^{++} showed nearly similar UA production and enzyme activity as shown in Table 7.

UA production using immobilized whole cell in sodium alginate

Entrapment of *P*. aeruginosa whole cells in varied concentration of calcium alginate beads (1, 1.5, 3 % w/v) was studied for production reinforcement. Calcium alginate immobilization was chosen because it entraps whole cells under mild conditions and causes minimal cell damage [14]. Initial enzyme activity decreased due to immobilization from 0.142 U/ml to 0.094 U/ml and simultaneously the UA production also (Table 8). The 1.5 % w/v sodium alginate concentration was determined to be the optimum concentration for efficient bioconversion (2.245 mg/ml) of histidine. The relative activity (%) measured in terms of decrease in initial UA production after 96 h, which clearly demonstrates the stabilization effect that immobilization has on the enzyme activity. Since an increase in the sodium alginate concentration would reduce the pore size of the calcium alginate beads, both conversion ratio and effluent cell concentration would also decrease. Klein et al. also reported that as the pore size of calcium alginate beads decreased, the mass transfer rate within the beads also decreased accordingly. Therefore, in case of 3 % w/v calcium alginate beads, due to reduction in bioavailability of substrate concentration inside the beads or transmission of oxygen and substrate to the organisms, the UA production was decreased up to 1.892 mg/ml. Only 8 % decrease in activity was displayed by the immobilized cells (1.5 % w/v sodium alginate) after incubation period as compared to the remained entrapped cells (34 % for 1 % w/v and 23 % for 3 % w/v sodium alginate).

Repetitive batch biotransformation for UA production

As previously mentioned one of the advantages of using immobilized biocatalysts is the prospect of their continuous or repeated use. In order to evaluate the possibility for repeated use, the same beads were reused in a batch bioconversion. Each batch reaction was conducted at 40 ^{0}C in a 100 ml column containing 80 ml of medium under aerobic conditions, up to 12 h. After one batch was finished, the calcium alginate beads were removed and washed using the sterilized distilled water and then inoculated into fresh reaction medium. The results revealed that, immobilized *P. aeruginosa* in calcium alginate beads could be reused up to five batches/cycles at which ~50% conversion of L-histidine to the UA production was attained (0.97 mg/ml).Until the 2nd and 3 rd batch bioconversion, 80 % (1.53 mg/ml) and 63 % (1.227 mg/ml) of the histidine conversion was attained. Thereafter, the histidine conversion decreased. A decrease in the bioconversion may be related with increase in pH due to ammonia production or histidine degradation that damage the calcium alginate beads or swelling of beads that prevent the transmission of substrate. The results are best summarized in Fig 2.

Storage stability of immobilized cells

The immobilized cells were stored in a 2 % w/v $CaCl_2$ solution at 4 ^{0}C for 8 weeks to investigate their storage stability. As per Fig 3, the UA produced at 12 h incubation after storage for 4 weeks that revealed only 8 % decrease in the previous production (1.642 mg/ml). With subsequent storage of the immobilized cells for 5 or 6 weeks the UA production decreased up to 25 or 37 % (1.35 or 1.12 mg/ml) from its initial value (1.79 mg/ml). A half value of the UA production was obtained at 7th week (0.892 mg/ml) later it decreased to 0.621 mg/mL. Therefore, *P. aeruginosa* cells entrapped in calcium alginate gels can be stored up to 4 weeks without noticeable loss of cellular activity.

In this report, we isolated *Pseudomonas aeruginosa* strain as promising UA producer from garden soil, confirmed and optimized at varied culture conditions to attain the maximum UA production (2.436 mg/ml) viz. histidine concentration 0.4 %, incubation period 96 h, pH 7.5, temperature 40 ^{0}C, 2% glucose and 0.5 % yeast extract as carbon and nitrogen source respectively. An enhanced UA production was achieved using 1.5 % w/v sodium alginate (2.245 mg/ml) when treated with histidine solution (0.25 mM). Furthermore, repetitive batch bioconversion and storage stability study revealed that the immobilized strain may be used up to five repetitive cycles and storing at 4 weeks at 4 ^{0}C temperatures retains 92 % conversion ability.

IV. CONCLUSION

From industrial point of view we demonstrate that the immobilized *P. aeruginosa* strain used in the bioreactor have the advantage of a good operational stability and efficiency of UA production. The future study is concentrated on optimization of culture conditions using immobilized cells and screening of other substances for immobilization.

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Table 1. Effect of histidine concentration on Urocanic acid production by isolated *Pseudomonas aeruginosa* species.

Histidine concentration (%)	Histidase activity (U/ml)	UA production (mg/ml)
0.1	0.044	0.33
0.2	0.052	0.65
0.3	0.051	0.92
0.4	0.046	1.25
0.5	0.045	1.02
0.6	0.035	0.86

Table 2. Effect of Incubation period on Urocanic acid production by isolated *Pseudomonas aeruginosa* species.

Incubation period (h)	Histidase activity (U/ml)	UA concentration (mg/ml)
24	0.031	0.89
48	0.036	1.05
72	0.044	1.18
96	0.052	1.23
120	0.048	1.15
144	0.046	1.14

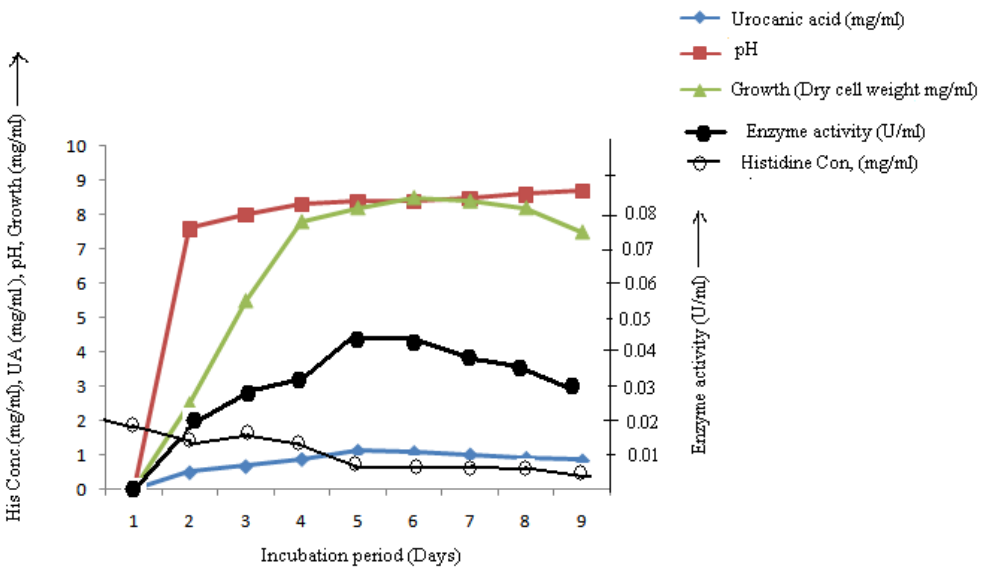

Figure 1. Changes during UA submerged production by *Pseudomonas aeruginosa* species.

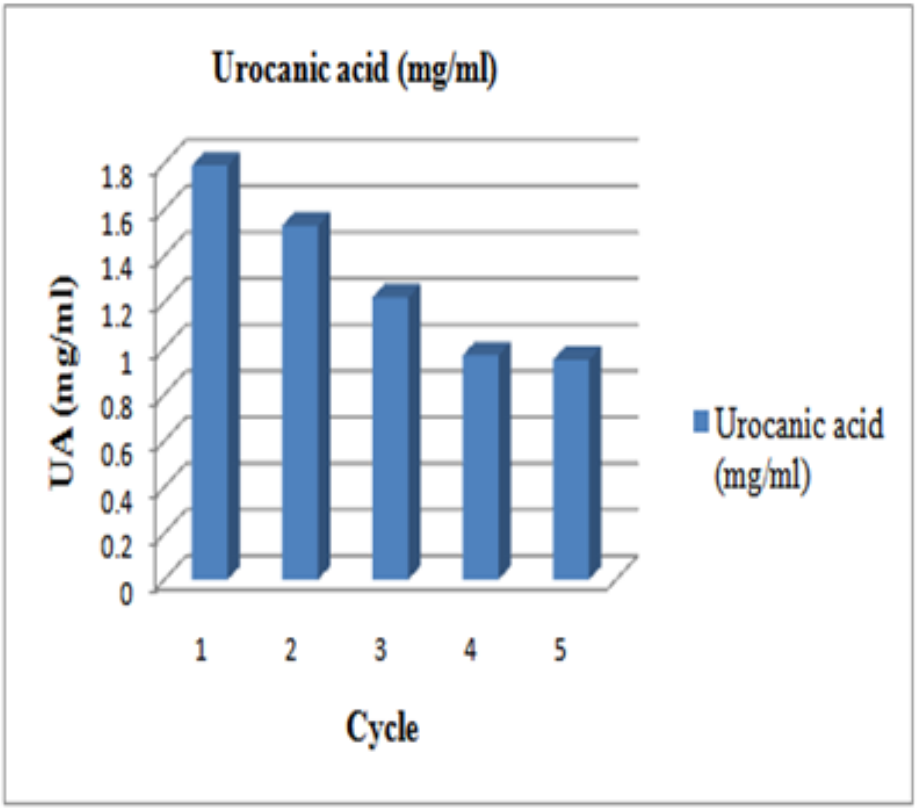

Figure 2. Repetative batch operation using 1.5 % sodium alginate

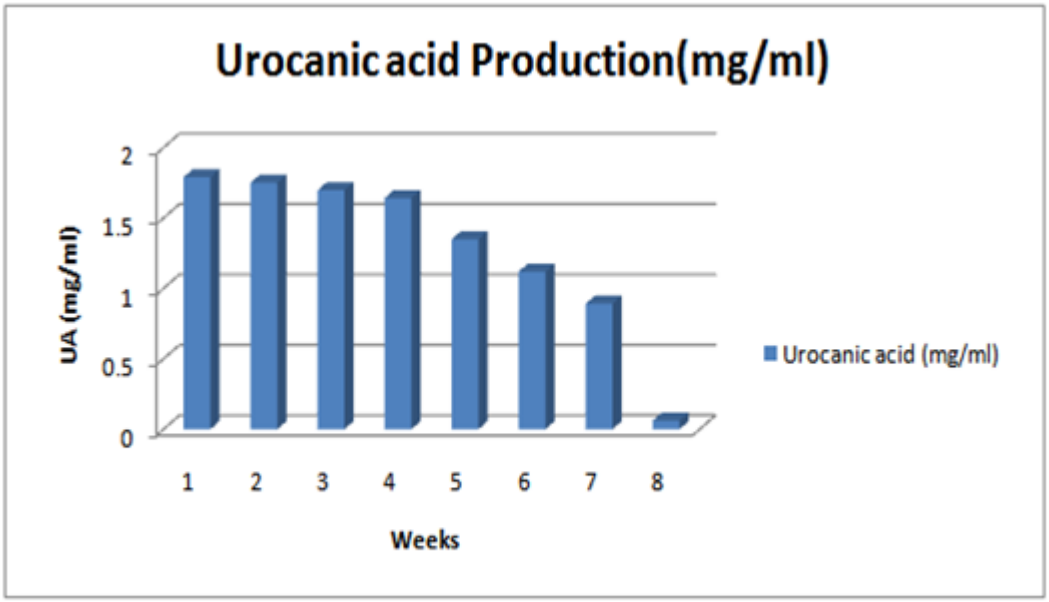

Figure 3. Storage stability of immobilized cells

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