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Bioconversion of Amino Acids to α-Keto Acid by *Geotrichum candidum* Used for the Treatment of Chronic Kidney Disorders

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A B S T R A C T

The present study explores the bioconversion by *Geotrichum candidum* of five amino acids to their corresponding α-keto acids used in the treatment of chronic kidney disorder. The amino acid oxidase activity biotransforming amino acids to α-keto acids was detected in biomass as well as extracellular culture fluids of *G. candidum.* Bioconversion was determined by measuring α-keto acid production along with residual amino acid. There was a 2-fold increase in the amount of α-keto acids produced over a period of 24 hours by permeabilization of biomass when compared to immobilized biomass. Permeabilized *G. Candidum* biomass was further immobilized by entrapment in Na-alginate and studied for the production of α-keto acids from the corresponding amino acids. The permeabilized and immobilized biomass beads showed an additional 2-fold increase in the α–keto acids production as compared to only permeabilized cells. Decomposition of hydrogen peroxide formed in the enzymatic reaction by manganese oxide incorporated in sodium alginate beads further improved the yield. The bioconversion efficiency of the studied amino acids to their corresponding keto acids obtained in the presence of MnO_2 by immobilized biomass of *G. candidum* at 4 h was found to be 29-34%.

Introduction

α-keto acids have been used in the therapy of certain conditions, e.g., uremia and nitrogen accumulation disorders. Uremia is the illness accompanying the kidney failure in particular the nitrogenous waste products associated with the failure of the organ. In kidney failure, urea and other waste products, which are normally excreted into urine, are retained in the blood. Keto analogues suppress urea formation by an amount of nitrogen equal to the stoichiometric quantity required to aminate them. Reduction of quantities of amino acids in circulation is expected to reduce urea synthesis. In this context, the keto analogues of the essential amino acids are of particular interest. The α-keto acid analogues of the naturally occurring amino acids are of major importance in intermediary metabolism, in the development of enzyme inhibitors and drugs, as model substrates of enzymes, and in other ways (Brodelius, 1978). Parikh *et al.*, (1958) provided an early example of the use of enzymes to prepare enantiomerically pure amino acids by selective destruction of one enantiomer in a racemic mixture with D- or L-amino acid

oxidase (D-/L-AAO). Amino acid oxidases are flavoproteins catalyzing the stereo specific conversion of amino acids to corresponding α–keto acids with ammonia and hydrogen peroxide as byproducts. In case of hog kidney DAAO, the use of crude, rather than pure enzyme preparation is recommended, as they possess residual catalase activity, whereas LAAO from snake venom has little or no catalase activity and the addition of catalase is necessary (Davis *et al.,* 2012). Amino acid oxidases are widely distributed in diverse organisms, from microorganisms to mammals.

Various organisms carry out enzymatic conversion of amino acids to α-keto acids as a part of amino acid metabolism. The cost of commercially available enzymes is high and therefore a suitable microbial source needs to be investigated. Industrial processes employ whole, permeabilized or crossed-linked cells or immobilized enzymes since these biocatalysts are re-usable, possess higher operational stability and require simple downstream processing of the products.

A number of researchers have worked on the bioconversion of amino acids to α-keto acids. Brodelius *et al.,* (1981) carried out the first work in the field where they immobilized the cells of *Trigonopsis variabilis* containing DAAO for the productionof α-keto acids. With the advancement in analytical and molecular techniques, the current scenario is such that D-/L-AAOis purified and recombinant strains are developed. These strains are used for increased enzyme production and also the enzymes are immobilized for α-keto acid production. Deshpande *et al.,* (1987) developed a method for the production of α-keto acids by coimmobilization of DAAO and catalase by entrapment of *T. variabilis* in radiation polymerized polyacrylamide beads. The operational half-life was of 7-9 days after which the DAAO activity remained stable at a value 35–40% of the initial activity for a study period of 3 weeks. Later Singh *et al.,* (2009) developed a shake flask technique for the production of LAAO by *Aspergillus fumigatus* with maximum enzyme production of 59 x 10^{-3}U/mg dry cell mass. Further the group has also purified and characterized the enzyme. One of the main problems of oxidases is the equimolar production of hydrogen peroxide as a side product that can inactivate them or alter its substrate.

The present study focuses on the bioconversion of amino acids to their corresponding keto acids by *Geotrichum candidum,* commonly used as starter culture in the production of cheese. To our knowledge this is the first report of biotransformation of amino acid to α-keto acids by *G. candidum*.

Materials and Methods

Culture conditions and preparation of biomass

The culture *Geotrichum candidum* was obtained from Enformtech, New Zealand. Commercially available reagent grade chemicals were purchased from HiMedia. Amino acids L-leucine, L- isoleucine, DL-methionine, L-valine and L-phenylalanine were used for α-keto acids production. *G. candidum* was grown in 25 ml GPY (1% glucose, 0.5% peptone and 0.5% yeast extract)in 100 ml Erlenmeyer flask incubated on rotary shaker (90 rpm, 20 h, 37˚C).

The activated culture was used to inoculate 400 ml GPY medium in 1000 ml Erlenmeyer flask and incubated (90 rpm, 19 h, 37˚C), and centrifuged (10,000 rpm, 10˚C, 20 min) to obtain biomass. Biomass obtained was washed thrice with phosphate buffer (50 mM, pH 8), and used in various experiments.

Bioconversion of amino acids to corresponding α-keto acids by *G. candidum*

Using untreated and permeabilized biomas

Untreated or permeabilized biomass (1 g wet weight)was added to the reaction mixture containing 29 ml phosphate buffer (50mM, pH 7) and 20 ml amino acids (50 mM).

Aliquots were collected every 4 h from the incubated (90 rpm, 37˚C) reaction mixture and analyzed for α-keto acid formed. Biomass (5 g wet weight) was permeabilized with 10 ml of permeabilization buffer (3 mM EDTA, 100 mM sucrose) for 30 min at 37˚C on rotary shaker at 90 rpm.

The cells were washed with phosphate buffer and used to set up the reaction mixtures.

Using extracellular fluid

Extracellular fluid (10 ml) was added to reaction mixture containing 20 ml phosphate buffer (50mM, pH 7) and 20 ml amino acids (50 mM). Aliquots were collected every 4 h from the incubated (90 rpm, 37˚C) reaction mixtures and analyzed for α-keto acid formed.

Using immobilized and permeabilized immobilized biomass

Untreated and permeabilized biomass was immobilized by entrapment in sodium-alginate gel. Cell suspension was prepared by adding 4 g of wet cells in 10 ml of phosphate buffer and 5 ml of this cell suspension was mixed with sterile 20 ml 2.5 % sodiumalginate and the mixture was subsequently added drop-wise into 50 mM $CaCl_2$.

The beads were collected after 1 h, by filtration and washed with Tris-HCl buffer (pH 7.5, 50 mM) containing 5 mM $CaCl_2$.

Using permeabilized immobilized biomass containing MnO_2

Sodium alginate containing manganese oxide (0.1%) was sonicated(60 amplitude x 2min, 4°C) using Ultraschall Homogenisator Lab Sonic (Probe 2 x 80mm, Sartorius, Labsonic M, Germany) for 5-6 cycles and centrifuged at 10,000 rpm, 15 min, 10°C) to disrupt the larger particles of MnO_2. The beads were prepared and used for bioconversion reaction as described above.

Bioconversion assay

Bioconversion assay was carried out according to Singh *et al* (2009) with minor modification. The amino acid oxidase activity of permeabilized cells was determined by measuring the amount of dinitrophenylhydrazine derivative of the keto acid produced. Briefly, 500 µl of 50 mM amino acid in sodium phosphate buffer (50 mM, pH 7.2) was mixed with 500 µl permeabilized biomassor extracellular fluid and incubated on rotary shaker (90 rpm) at 37°C for 60 min. The reaction was terminated by adding 450 µl of 20% trichloroacetic acid and held at room temp for 30 min.

The amino acid oxidase activity of immobilized cells was determined by suspending the immobilized cells (0.2 g wet beads) in 5 ml of 50 mM amino acid in sodium phosphate buffer (50 mM, pH 7.2) and incubated on rotary shaker (90 rpm, 37°C, 60 min). The reaction was terminated by adding 450 µl of 20% trichloroacetic acid and held at room temp for 30 min. This was further analyzed to determine the amount of keto acid produced.

Estimation of keto acid produced in the bioconversion reaction assays

The sample (500 µl) was mixed with 2,4-dinitrophenyl hydrazine (2,4-DNPH) in 2 M

HC1 (0.2 ml). After l0 minutes, 700µlof 3M NaOH was added and 15 min later the reaction mixture was centrifuged and A_{550} was determined. Pyruvate was used as the standard. The reaction mixture without amino acid was used as control.

Estimation of residual amino acids

Residual amino acid content was determined largely according to Lee and Takahashi (1966) with minor modification.1%Ninhydrin (in ethanol) was added to 500 µl of bioconversion reaction mixture, tubes were covered with aluminum foil, incubated in water bath (100ºC, 10 min) and cooled immediately. A_{570} was measured using UV-Visible spectrophotometer (UV 1601, Shimadzu, Japan).

Results and Discussion

Bioconversion of amino acids to corresponding α–keto acid

Using biomass

*G. candidum*shows the bioconversion of L-valine, DL-methionine, L-phenylalanine, L-isoleucine and L-leucine to corresponding α–keto acids (Figure 1). The amount of α–keto acid production increased over time up to 24 h for all amino acids. Highest bioconversion was observed for methionine.

Using extracellular fluid

G.candidum produced and secreted amino acid oxidase activity in the medium, which increased with incubation time of 24 h. The amount of α-keto acids produced increased up to 24 hours (Figure 2). Bioconversion of methionine to this corresponding keto acid was again observed to be higher than the other amino acids.

Effect of immobilized, permeabilized and permeabilized immobilized biomass

The permeabilized immobilized biomass beads showed 2-fold increase in the α–keto acid production as compared to only permeabilized cells. The least bioconversion was observed with immobilized whole cells (Figure 3).

Effect of permeabilized biomass immobilized with MnO_2

Immobilization of permeabilized biomass in sodium alginate containing MnO_2 showed further 2.5-fold increase in bioconversion ability by *G. candidum* as compared to beads without MnO_2 (Figure 4). Similar results were found by Deshpande *et al.*, (1987) wherein the initial rates of conversion in the MnO_2 columns were about 1·5 times higher than those without MnO_2.

Residual amino acid

The amount of amino acid decreased gradually over the time (Figure 5).

Bioconversion efficiency

The conversion efficiency was calculated on the basis of amino acids biotransformed and the amount of keto acid produced. The conversion efficiency was found to be 2 fold higher in L-leucine and L-isoleucine. It was 3 fold higher in L-phenylalanine and 4 fold higher for DL-valine.

α-keto acids are of continuing interest as intermediates in chemical synthesis, in the development of enzyme inhibitors and drugs, as model substrates of enzymes, and in other ways. Patients suffering from acute uremia have a positive nitrogen balance and thus an excess of blood nitrogen that must be reduced (Table 1).

Table.1 Bioconversion of amino acids to α-keto acid produced by permeabilized and immobilized biomass of *G. candidum* biomass with MnO_2 after 4 h at 37°C

	with MnO_2				without MnO_2
Amino acid	**Amino acid added (nmoles/ml)**	**α-keto acid produced (nmoles/ml)**	**residual amino acid (nmoles/ml)**	**% Conversion**	**% Conversion**
L-Leucine	20	5.1	4.2	32	16
DL-Methionine	20	4.7	4.6	28	17
DL-Valine	20	4.4	5.0	43	10
L-Isoleucine	20	5.4	4.3	32	17
L-Phenylalanine	20	4.4	5.4	37	12

Figure.1 Bioconversion of valine, methionine, phenylalanine, isoleucine and leucineto corresponding α– keto acids (◆α-ketovaline, × α-ketomethionine, + α-keto phenylalanine, ■α-keto isoleucine and ●α-keto leucine) at every 4 h, 37°C by *G. candidum* biomass

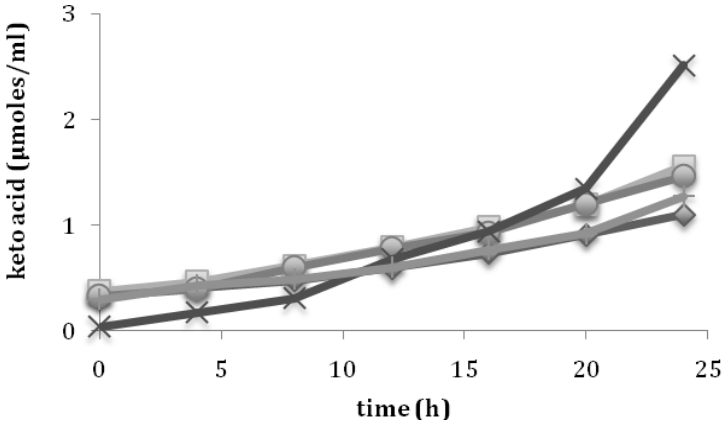


Figure.2 Bioconversion of valine, methionine, phenylalanine, isoleucine and leucine to corresponding α– keto acids (◆α-ketovaline, × α-ketomethionine, + α-keto phenylalanine, ■α-keto isoleucine and α-keto leucine) at every 4 h, 37°C by extracellular fluid produced during growth of *G. candidum* biomass.

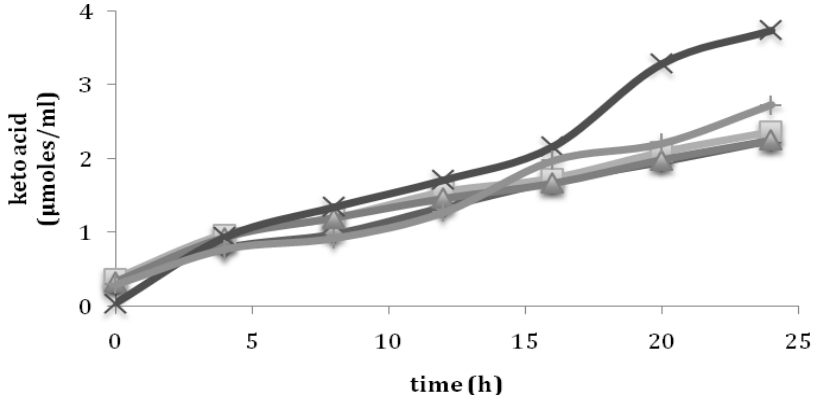


Figure.3 Bioconversion of valine, methionine, phenylalanine, isoleucine and leucine to corresponding α– keto acids at 24 h, 37°C by immobilized, permeabilized biomass and permeabilized-immobilized biomass of *G. candidum*

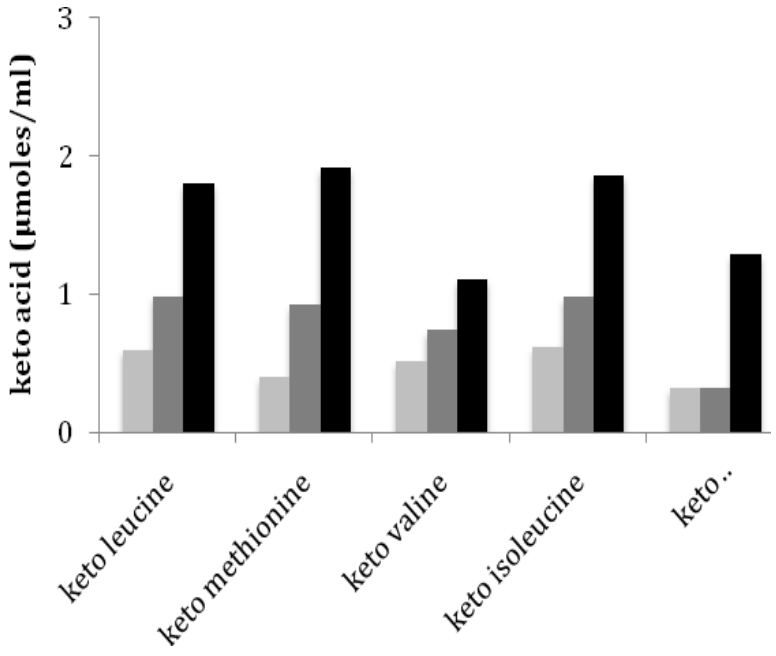


Figure.4 Bioconversion of amino acids to corresponding α–keto acids by *G. candidum* biomass permeabilized and immobilized in sodium alginate beads in the presence (■) and absence (□) of MnO_2

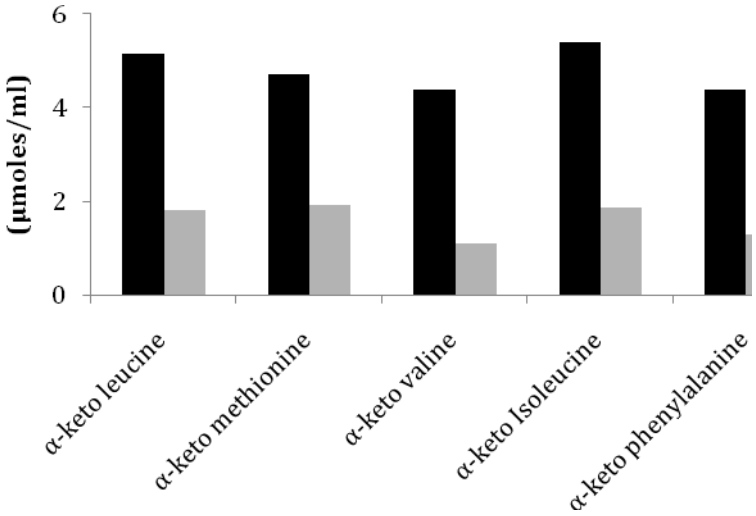
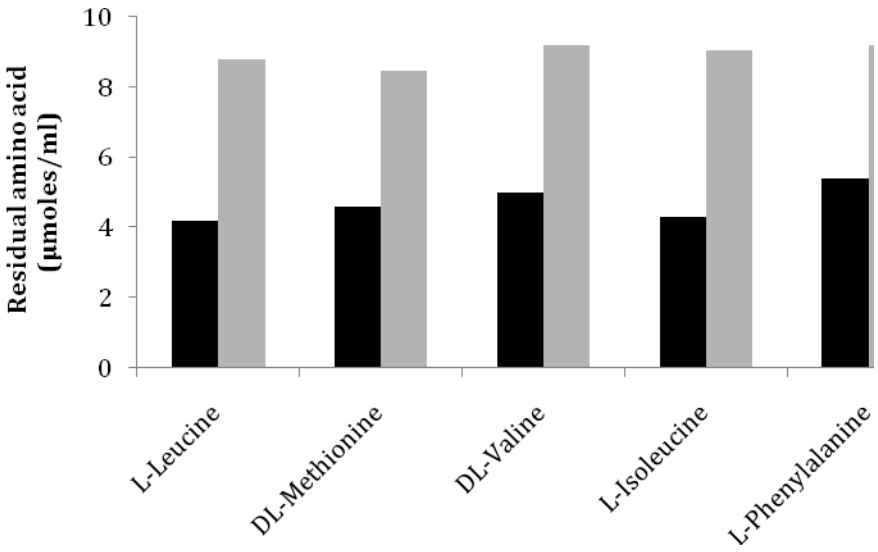


Figure.5 Residual amino acids during the bioconversion to corresponding α-keto acids by *G. candidum* biomass immobilized in sodium alginate beads in the presence (■) and absence (■) of MnO_2



This is achieved in an initial state of the disease, before dialysis is required, by administration of a diet containing low protein and high carbohydrate content (Bergstorm *et al.,* 1972). Keto analogues of valine, isoleucine, methionine, phenylalanine and leucineare used as supplementary nutrients for the treatment of chronic ureamia and other kidney disorders. Ketolog is the drug manufactured by Claris Life sciences, India commercially available for treatment in chronic renal insufficiency.

The chemical synthesis of some of these analogues is not possible on a commercial basis. There are few keto acids where synthetic route has very less yields and prohibitory chemicals like acetic anhydrous are used which prevent, commercially viable and environmentally benign, process development. Enzymatic conversion of amino acids to α-keto acids has been carried out by various organisms as a part of amino acid metabolism. The cost of commercially available enzymes is high and therefore we used *Geotrichum candidum,* a filamentous fungus of industrial importance. *G. candidum* is generally used as a starter culture in dairy industry for the production of cheese (Jollivet *et al.,* 1994), and source of lipase (Bertolini *et al.,* 1995).

Present study shows that *G. candidum* is able to efficiently biotransform these amino acids to corresponding α-keto acids. Intracellular amino acid oxidases involved in biotransformation of amino acids to α-keto acids are located in the peroxisomes. Such localization of the enzyme provides the efficient removal of the cell toxicant, hydrogen peroxide, produced in the course of DAAO catalyzed reaction (Preston, 1987). Thus permeabilization of the cells becomes essential. Brodelius *et al.,* (1981) have shown the feasibility of immobilizing this enzyme in Ca-alginate using intact cells. This research results established that the permeabilized biomass biotransformed higher amount of amino acids to corresponding α-keto acid over a period of time. The immobilized cells are

advantageous as they can be reused and are easy to handle. The permeabilized immobilized cells show higher bioconversion ability as compared to immobilized whole cells and permeabilized biomass. Hydrogen peroxide (H_2O_2) is formed in the enzymatic reaction of conversion of amino acid to α-keto acids. The requirement for the efficient degradation of this product is one of the main problems in developing a process for the production of keto acids. Hydrogen peroxide denatures proteins and, therefore, influences the operational stability of the immobilized biocatalyst. A secondary reaction between hydrogen peroxide and α-keto acids can cause acid decarboxylation lowering the yield of α-keto acids (Messing, 1974). Decomposition of hydrogen peroxide leading to the formation of oxygen can favorably influence the reaction rate.H_2O_2-degrading agents like MnO_2 and activated charcoal improve the operational stability (Brodelius *et al.,* 1981; Szwajcer *et al.,* 1982). The immobilization of permeabilized biomass pellet and MnO_2 increased the keto acid production by 2 fold. The storage stability of immobilized DAAOin *T. variabilis* with MnO2 nearly doubled and production of 2-oxoadipyl-7-aminocephalosporanic acid was 2-3-fold higher than by entrapped cells without MnO_2 (Vikartovská-Welwardová, 1999). The efficiency of conversion of all five studied amino acids to their corresponding keto acids obtained in the presence of MnO_2 by immobilized biomass of *G. candidum* at 4 h was found to be 29-34%. This process can further be optimized to increase the yield by studying the kinetic parameters.

Safety is an important aspect to be considered when considering the product for treatment. *G. candidum* does not appear on the official list of biological agents published by the Advisory Committee on Dangerous Pathogens (2004). Thus there is no adverse effect on health when using *G. candidum* for the production of keto analogues of amino acids.

In conclusion, the present study establishes the ability of *G. candidum* to biotransform five amino acids to corresponding α-keto acids, which are used as supplementary nutrients in the treatment of chronic kidney disorders. Immobilization of permeabilized biomass in sodium alginate beads along with MnO_2 efficiently increases the bioconversion ability. Further work includes study of the kinetic parameters to develop this method for the production of α-keto acid on a larger scale.

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