

Production of L-Arginine Using Wastes of *Carica Papaya* and *Ananas Comosus* by *Pediococcus Pentosaceus*

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ABSTRACT: The present study was an illustrative investigation on the production of one of the essential amino acid L-arginine using two different wastes (*Carica Papaya* and *Ananas Comosus*) as substrates. An aerobic fermentative process was followed for the production by the bacteria *Pediococcus Pentosaceus*. The optimal characteristics temperature, fermentation time and pH for the maximum production were assayed. The quality of L-arginine was analysed by Kossel method and Arginine Dihydrolase Test and quantitative estimation was done by using ninhydrin reagent. From the investigations, an optimum temperature at 30°C with pH 9 and aeration were found to be suitable for max production in 5 days using both the substrates (0.54 mg/ml) (0.675 mg/ml) waste media.

KEYWORDS: L-arginine, *Pediococcus Pentosaceus*, *Carica Papaya*, *Ananas Comosus*, Kossel method, Arginine Dihydrolase Test, Ninhydrin method.

I. INTRODUCTION

L-Arginine is an important component of drugs for stimulating liver functions, amino acid infusion, total amino acid preparations, etc. To reduce the production costs of L-arginine, it is important to improve the fermentation yield. In order for microorganisms belonging to the genus *Brevibacterium* or the genus *Corynebacterium* to exhibit L-arginine productivity, it is known that a resistance to Z-thiazolealanine, arginine hydroxamate, etc. should be imparted to the microorganism. The production of L-arginine is improved by imparting sulfa agent or argininol resistance, resistance to chemicals such as 8-azaguanine, a-amino-B-hydroxyvaleric acid, etc., in addition to the chemical resistance, and by imparting auxotrophy for amino acids such as L-histidine, L-proline, L-threonine, L-tryptophan, L-lysine, etc.

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However, use of the conventional L-arginine-producing strains of microorganisms results in an unsatisfactory yield of L-arginine. Thus, there remains a need for a process for producing L-arginine by fermentation in high yield. There also remains a need for strains of microorganisms which produce L-arginine in high yield by fermentation.

L-Arginine excretion by a canavanine resistant mutant of *Escherichia coli* (G. M. Peru *et al.*, 1967) or *Saccharomyces cerevisiae* (F. Ramos *et al.*, 1971) has been reported. More recently, it has been reported that the arginine hydroxamate-resistant mutant of *Bacillus subtilis* (M. Kisumi *et al.*, 1971) produced L-arginine. However, the amounts of the L-arginine produced by these mutants seem to be too small for the industrial production of L-arginine. Accordingly, the development of a more effective fermentative method for L-arginine production was attempted.

L-arginine is an amino acid classified as essential with respect to its growth effect in rats. The amino acid is also useful *inter alia* as a starting compound in the preparation of arginine glutamate which is an adjunct in management of ammonium intoxication due to hepatic failure. Here, L-arginine has been prepared using various methods. For example the L-form has been obtained by hydrolysis of proteins. In industrial practice it is precipitated from gelatine hydrolyzate as the Flavianate. However, natural processes which have a high yield of L-arginine are in demand for utilization in industrial practice (C. J. Weber *et al.*, 1930).

Most of wild strains of microorganisms do not produce L-arginine in the medium. In order to render a wild strain capable of producing L-arginine from carbohydrates, it has been necessary to induce artificial mutants from the wild strain. There are many known arginine - producing artificial mutants. It has however, become difficult to increase the yields of L-arginine by using the artificial mutation techniques. Therefore, a continuous need exists for the development of novel method for producing L-arginine in high yields (E. S. Talor *et al.*, 1945).

Canavanine is a non-proteinogenic α -amino acid found in certain leguminous plants. It is structurally related to the proteinogenic α -amino acid L-arginine, the sole difference being the replacement of a methylene bridge (-CH₂- unit) in arginine with an oxa group (i.e., an oxygen atom) in canavanine. Canavanine is accumulated primarily in the seeds of the organisms which produce it, where it serves both as a highly deleterious defensive compound against herbivores and a vital source of nitrogen for the growing embryo⁵. The mechanism of canavanine's toxicity is that organisms that consume it typically mistakenly incorporate it into their own proteins in place of L-arginine, thereby producing structurally aberrant proteins that may not function properly (Rosenthal *et al.*, 1982; Rosenthal *et al.*, 1986).

L-Arginine was first isolated from a lupine seedling extract in 1886. It was identified as a component of casein in 1895; later it was found to be widely distributed in foods and feed. L-Arginine is broken down by arginase to form L-ornithine and urea in mammals, completing the ammonia excretion system. L-Arginine is recognized as performing an important function in mammals. The production methods for amino acids are summarized as follows: 1) protein hydrolysis, 2) chemical synthesis, and 3) microbiological synthesis. Most L-arginine has been produced by the direct-fermentation method from natural carbon sources (Yoshida, H, 1986; Kubota *et al.*, 1973; Momose *et al.*, 1982; Akashi *et al.*, 1979; Akashi *et al.*, 1979).

L-arginine is an industrially useful amino acid as ingredient of liver function promoting agents, transfusion solutions, food additives and the like. In microorganisms, biosynthesis of L-arginine proceeds in eight enzymatic steps starting from the precursor L-glutamate and follows two different pathways, the linear pathway or the cyclic acetyl pathway depending on the microorganism concerned (Cunin *et al.*, 1986; Davis, 1986). In both biosynthetic pathways the first step is N-transacetylation of glutamate catalysed by the enzymes displaying N-acetyl glutamate synthase activity. In the linear pathway, the acetyl glutamate synthase activity is provided by the enzyme acetyl-coA: L-glutamate N-acetyltransferase encoded by the *argA* gene and in this pathway the intermediate N-acetyl L-ornithine is converted into L-ornithine at the fifth enzymatic step through deacetylation by N₂-acetyl-L-ornithine amidohydrolase encoded by the *arg E* gene.

There has been substantial examination of the effect of infusion and ingestion of L-arginine at rest. It has been clearly demonstrated that L-arginine administration improves endothelial function in various disease states. In addition, L-arginine infusion at rest increases plasma insulin, growth hormone, glucagon, catecholamine's and prolactin. Such

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hormonal changes affect metabolism. There has, however, been very little examination of the effect of increases in L-arginine availability during exercise. This is important to study as there is preliminary evidence that L-arginine infusion, probably via increases in nitric oxide (NO), alters skeletal-muscle metabolism during exercise.

Raw pineapple is an excellent source of manganese (76% Daily Value (DV) in a one US cup serving) and vitamin C (131% DV per cup serving). Mainly from its stem, pineapple contains a proteolytic enzyme, bromelain, which breaks down protein. If having sufficient bromelain content, raw pineapple juice may be used as a meat marinade and tenderizer. Pineapple enzymes can interfere with the preparation of some foods, such as jelly or other gelatine-based desserts, but would be destroyed during cooking and canning. The quantity of bromelain in the fruit is probably not significant, being mostly in the inedible stalk. Furthermore, an ingested enzyme like bromelain is unlikely to survive intact the proteolytic processes of digestion.

Traditional medicine and preliminary research:

Both the root and fruit may be eaten or applied topically as an anti-inflammatory or as a proteolytic agent. In some practices, it may be used to induce abortion or menstruation or as an anti-helminthic agent. Bromelain purified from pineapple stem or fresh juice, then provided in the diet over six months, decreased the severity of colonic inflammation in mice with experimental colitis. Bromelain from pineapple has some potential against cancer mechanisms, as laboratory research showed that it causes autophagy in mammary carcinoma cells, stimulating turnover of MCF-7 cells through apoptosis (**Definition of Pineapple at Dictionary.com, 2009**).

Pineapples are consumed fresh, cooked, juiced, and preserved, and are found in a wide array of cuisines. In addition to consumption, in the Philippines the pineapple's leaves are used to produce the textile fibre piña- employed as a component of wall paper and furnishings, amongst other uses. In the scientific binomial *Ananas comosus*, ananas, the original name of the fruit, comes from the Tupi word nanas, meaning "excellent fruit", as recorded by André Thevet in 1555, and *comosus*, "tufted", refers to the stem of the fruit (**Coppens et al., 2003**).

The papaya is a large, tree-like plant, with a single stem growing from 5 to 10 m (16 to 33 ft) tall, with spirally arranged leaves confined to the top of the trunk. The lower trunk is conspicuously scarred where leaves and fruit were borne. The leaves are large, 50–70 cm (20–28 in) in diameter, deeply palmately lobed, with seven lobes. Unusually for such large plants, the trees are dioecious. The tree is usually unbranched, unless lopped. The flowers are similar in shape to the flowers of the *Plumeria*, but are much smaller and wax-like. They appear on the axils of the leaves, maturing into large fruit - 15–45 cm (5.9–18 in) long and 10–30 cm (3.9–12 in) in diameter. The fruit is ripe when it feels soft (as soft as a ripe avocado or a bit softer) and its skin has attained amber to orange hue (**Oxforddictionaries.com, 2013**).

Both green papaya fruit and the tree's latex are rich in papain, a protease used for tenderizing meat and other proteins. Its ability to break down tough meat fibres was used for thousands of years by indigenous Americans. It is now included as a component in powdered meat tenderizers.

Papaya fruit is a source of nutrients such as provitamin A carotenoids, vitamin C, folate and dietary fibre. Papaya skin, pulp and seeds also contain a variety of phytochemicals, including lycopene and polyphenols. In preliminary research, danielone, a phytoalexin found in papaya fruit, showed antifungal activity against *Colletotrichum gloeosporioides*, a pathogenic fungus of papaya.

The black seeds of the papaya are edible and have a sharp, spicy taste. They are sometimes ground and used as a substitute for black pepper. In some parts of Asia, the young leaves of the papaya are steamed and eaten like spinach (**Merriam-webster.com, 2013**).

Papaya is marketed in tablet form to remedy digestive problems. Papain is also applied topically for the treatment of cuts, rashes, stings and burns. Papain ointment is commonly made from fermented papaya flesh, and is applied as a gel-like paste. Harrison Ford was treated for a ruptured disc incurred during filming of *Indiana Jones and the Temple of Doom* by papain injections (**Boning et al., 2006**).

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In some parts of the world, papaya leaves are made into tea as a treatment for malaria. Anti-malarial and anti-plasmodial activity has been noted in some preparations of the plant, but the mechanism is not understood and no treatment method based on these results has been scientifically proven. In belief that it can raise platelet levels in blood, papaya may be used as a medicine for dengue fever. Papaya is marketed in tablet form to remedy digestive problems (Gizachew Yismaw *et al.*, 2008).

II. MATERIAL AND METHODS

Pediococcus Pentosaceus strains was obtained from NCIM Pune collection centre with accession number 2295. *Pediococcus Pentosaceus* was cultured on standard MRS plate, pure cultures are obtained from single colonies and maintained on agar slants at 4 °C till use.

a) Preparation of *Pediococcus Pentosaceus* inoculums:

Pediococcus Pentosaceus was cultured on standard MRS broth before inoculating to the production media at 25 °C for 48 hrs.

b) Arginine production testing with *Pediococcus Pentosaceus*:

Ability of *Pediococcus Pentosaceus* to produce Arginine was assayed by using standard production medium contains (protease Peptone - 1g; Yeast extract - 0.5g; Beef extract - 1g; Dextrose - 2g; Tween 80 - 0.02ml; Ammonium citrate - 0.2g; Sodium acetate - 0.5g; Magnesium sulphate - 0.05g; Manganese sulphate - 0.005g; Dipotassium phosphate - 0.2g) in 1000 ml distilled water. Media was sterilized by using autoclave at 121.1 °C and 15 lbs. pressure for 15 min and cooled to 25 °C. 1% v/v *Pediococcus Pentosaceus* inoculum was added to the media and fermented for 5 days. Broth was filtered in sterile conditions and arginine released into the media was qualitatively assayed by Arginine Dihydrolase Test and Kossel test (Arginine Flavianate) and quantitatively assayed by Ninhydrin method.

c) Preparation of Media with papaya and pine apple wastes:

A 50gms of *Ananas comosus* peel paste and *Carica papaya* peel paste were taken separately. To this pastes, mineral salts (Urea-0.8g; KH_2PO_4 -0.08g; K_2HPO_4 -0.08g; MgSO_4 -0.04g; MnSO_4 -0.001g; FeSO_4 -0.001g; Biotin-1 μg) were added to 1000 ml of distilled Water. Production media was sterilized by using autoclave at 121.1 °C and 15 lbs pressure for 15 min. Production media was cooled to 25 °C and inoculated with 1% v/v *Pediococcus Pentosaceus*. For production of more quantity of arginine, temperature and pH of the media was optimized by fermenting media at different temperatures (20°C, 30°C, 40°C and 50°C), pH (3, 5, 7, 9 and 11) and fermented time (1, 2, 3, 4 and 5 days). After 24, 36, 48, 60 and 72hrs from initiation of fermentation, 5 ml of broth was collected and assayed for arginine production.

d) Arginine Dihydrolase Test:

Arginine Dihydrolase broth and fermented broth was prepared and pH of the broth was maintained at 6.0 \pm 0.2 then added with loop full of *Pediococcus Pentosaceus* culture and incubated with at 35°C in bacteriological incubator for 24 - 48 hrs. Bromo-cresol blue was added to broth as indicator. Production of arginine Dihydrolase by bacteria and presence of arginine in solution was confirmed by observing change of colour of broth from purple to yellow and back to purple.

e) Kossel (Arginine Flavianate) test:

25 Gms of gelatine was fluxed with 250ml of 18% HCl for 18 hrs. at RT and excess HCl was removed by repeated concentration in vacuum. Residue was added with 250ml of hot water and 5g active charcoal was added for

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decolorize. Filtrate was added with 200gm of flavianic acid (2,4-dinitro-1-naphthol-7- sulponic acid) at room temperature and settled for 5 days to form yellow precipitate. Yellow precipitate was collected by filtering with Whatman No 1 filter paper. Yellow precipitate was washed with cold double distilled sterile water and dissolved the Yellow precipitate in hot water under 4% of ammonia. Ammonia was neutralized with 20 % HCl. Shining yellow crystals (Arginine Flavianate) formed in the hot solution indicates positive for arginine production.

f) Quantitative estimation of arginine by Ninhydrin Method:

Clean and sterile test tubes were taken. Add 0.1ml to 1.0 ml with liner increase of 0.1 ml of standard arginine stock solution along with 1ml of test sample; with no standard arginine solution as blank or control. And make up to 4 ml with sterile distilled water. Add 1ml of ninhydrin solution to all test tubes and incubate in water bath at 80-100°C in water bath for 15 min, then tubes were cooled to RT and added with 1 ml of ethanol and vortexed. By using colorimeter, OD of the samples was recorded at 570nm. Concentration of the unknown sample was calculated using formula. Graph was plotted and concentration of test solution was plotted from the graph.

$$\text{Conc. of test sample (mg)} = \frac{\text{OD of test}}{\text{OD of Standard}} * \text{Conc. of the Standard Sol. OD}$$

III. RESULTS AND DISCUSSION

This study was mainly carried out to identify the important components that could possibly assign some new uses to the fruit peel management rather than them being disposed of as solid wastes. In the present study, papaya and pineapple waste (peel) were tested for the maximum production of arginine using *Pediococcus Pentosaceus*.

i. Arginine Dihydrolase Test:

Yellow colour broth was observed in both pineapple and papaya waste media with bromo-cresol as indicator. Yellow crystals indicating Arginine Flavianate was also observed in both pineapple and papaya waste media indicating arginine was produced in both media.

ii. Optimization of temperature, P^H and time of fermentation for production of Arginine

Fermenting broth at different temperatures, pH and for fermentation for 5 days it was observed that temperature and pH makes considerable variation in production of arginine. Only at 30 °C (0.32, 0.30) production of arginine is observed form both media (pineapple and papaya) and remaining temperatures 20(0.24, 0.24) > 40(0.22, 0.20) and low 50(0.19, 0.18) arginine production was observed. Different quantity of arginine was produced in both media (pine apple and papaya). Optimal production of arginine from papaya waste media was observed at pH 9(OD: 0.3), followed by pH11 (0.27) > pH 3(0.22) > pH 5(0.18) and low production at neutral pH7 (0.14). Optimal production of arginine from pine aapplewaste media was observed at pH 9(OD: 0.22), followed by pH 7 and pH11 (0.20) > pH 5(0.18) > and low production at pH 3(0.17). In both media (pineapple and papaya) pH 9 was most suitable for high production of arginine. Media prepared from pineapple and papaya wastes showed maximum production of arginine by fermenting for 5 days. From this optimum temperature 30 °C at pH 9 for 5 days produced maximum arginine from both pineapple and papaya is used for fermentation.

iii. Effect of P^H on production of Arginine

The influence of P^H was studied carefully on the yield of arginine and that indicates the optimum concentration of arginine for papaya and pineapple was recorded at P^H9. The results are shown in the below figure.

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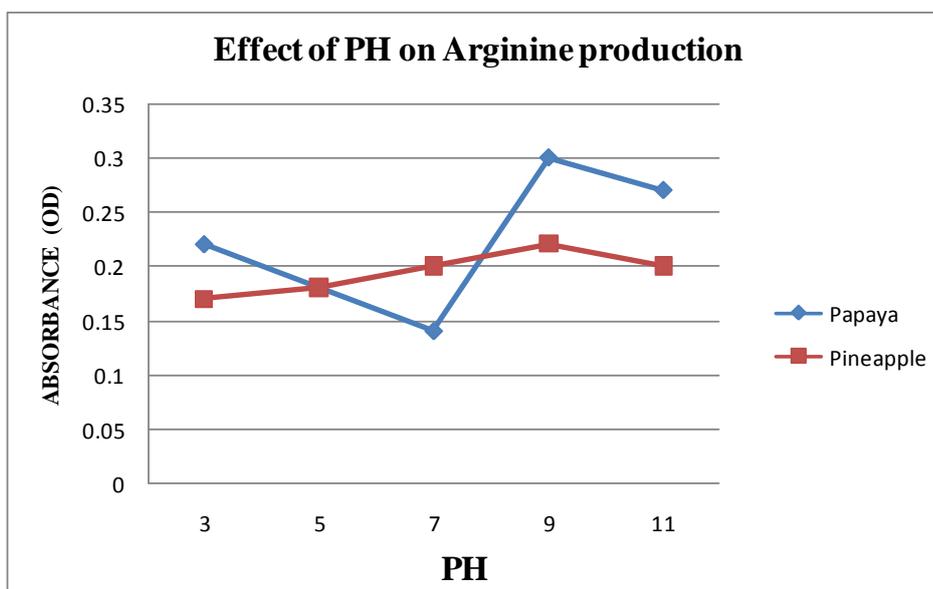


Fig. 1. Effect of P^H on arginine production: P^H 9 was found suitable for the production of arginine, which shows maximum yield for both pine apple and papaya.

iv. Effect of Temperature on production of Arginine

The production of Arginine was tested under various temperatures and the optimum temperature for both papaya and pineapple was found to be 30⁰C for maximum production of Arginine. The results are shown in the below figure.

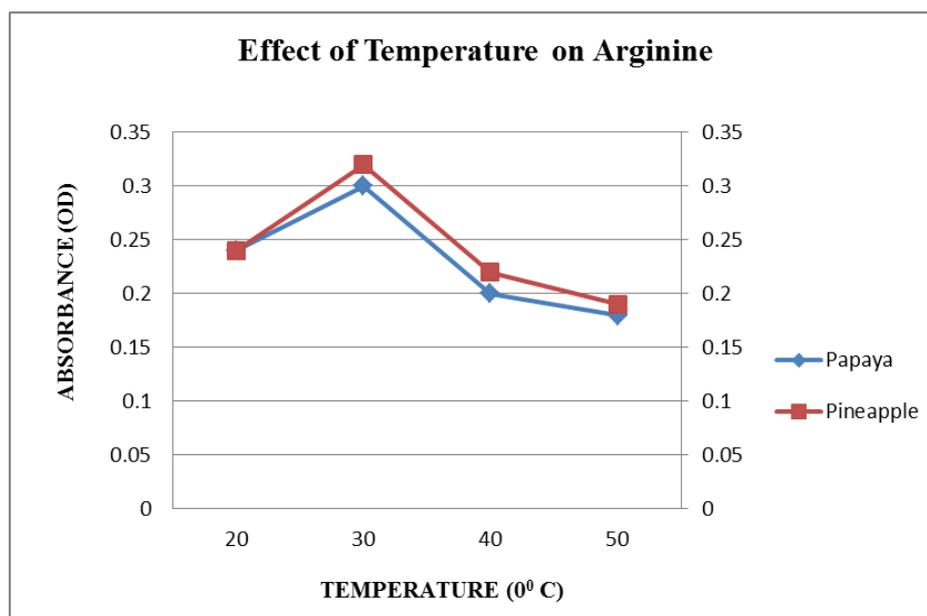


Fig. 2. Effect of Temperature on arginine production: 30⁰C was found optimum temperature for the production of arginine for both papaya and pine apple.

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v. Quantative estimation of arginine by Ninhydrin Method

Fermentation was carried out at optimal temperature 30 °C at pH 9 for 5 days and broth and arginine crystals obtained were estimated with standard arginine graph. Maximum yield of arginine production was obtained (0.54 mg/ml) in pineapple waste and (0.675 mg/ml) in papaya waste respectively.

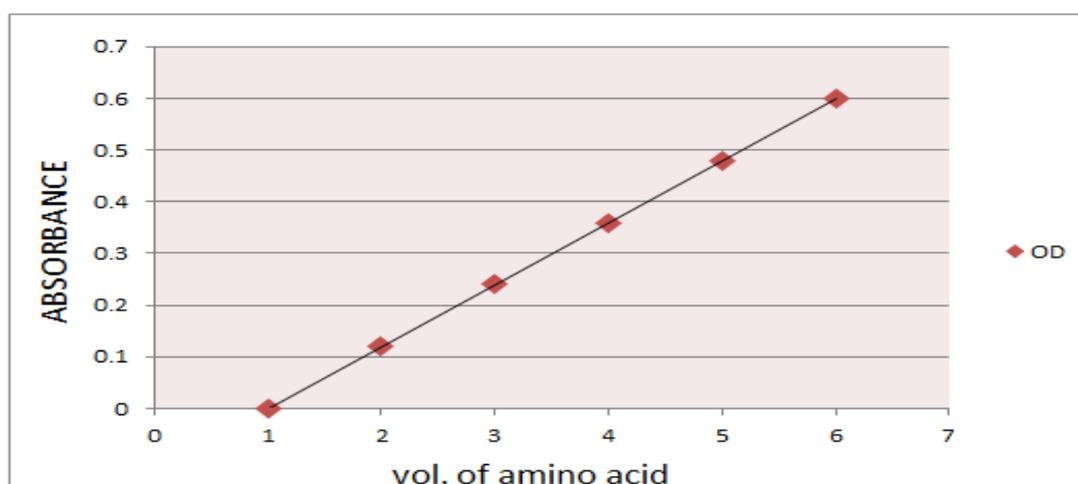


Fig. 3. Standard graph of L – Arginine using Ninhydrin method:

IV. CONCLUSION

The investigation report on arginine production using *Pediococcus pentosaceus* under submerged fermentation conditions, the fruit wastes were suitable for the production of arginine. Various factors like incubation period (5 days), pH (9), temperature (30 °C) were studied extensively using *Pediococcus pentosaceus* and the yield was 675 mg/1000ml and 540 mg/1000 for papaya and pineapple waste respectively.

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