Mechanism of 2-Acetyl-1-Pyrroline Biosynthesis by Bacterial System

Yogita Deshmukh[1*], Puja Khare[1] D.D. Patra[1]

[1]Agronomy and Soil Science Division, CSIR-CIMAP, Lucknow, India
[2]Department of Botany, University of Pune, Pune – 411007, India

Abstract- The biological formation of 2AP, by several rhizobacterial isolates was studied on the medium supplemented with L-ornithine. Increase in concentration of 2AP, was observed when proline (4%), ornithine (4%) and putrescine (5%) were supplemented in growth medium. *Acinetobacter bahayai*-BM-28- KJ143625 was a highest 2AP producing isolate. Medium inoculated with different rhizobacterial isolates was observed with 2-15.4 fold increase over the control. *A. bahayai*-BM-28- KJ143625 was inoculated in the medium supplemented with different concentrations of 4-aminobutanal and 1-pyrroline. 2-AP was identified as the most important volatile found in the headspace of samples supplemented with different concentrations of 4-aminobutanal before sterilization. 1-pyrroline was found as one of the very important immediate precursor for 2AP synthesis by bacteria. Results of precursor analysis revealed that 2AP production occurs when 4-aminobutanal diethyl acetal deduced from ornithine/proline/putrescine is heat transformed to 1-pyrroline which ultimately is converted into 2AP by bacterial system.

Index Terms- 2-acetyl-1-pyrroline/precursors/ Basmati rhizobacteria/ ornithine

Running title: 2-acetyl-1-pyrroline biosynthesis

I. INTRODUCTION

Microorganisms are essential part of flavor industry. The products/flavors produced by microbial *de- novo* production or bioconversion of natural precursors leads to flavoring substances that can be labeled as 'natural' and represent an interesting area in the world of food science. Various studies reported the microbial origin of 2-acetyl-1-pyrroline (2AP), a pleasant popcorn like/pandan like aroma compound. *Bacillus cereus* strains, LABs like *Lactobacillus hilgardii* and *Lactobacillus brevis*, fungal strains *viz Acremonium nigricans*, *Aspergillus awamori*, and *Aspergillus oryzae* and a yeast strain, *Kluveromyces maxicans* var. marxianus (Hansen) and *Saccharomyces cervisiae* were reported as 2AP producers (Adams and De Kimpe 2007; Costello and Henschke 2002; Shaikh and Nadaf 2013)

Since the discovery of 2AP in variety of sources the accumulation of 2AP, the principle basmati aroma compound in cereals, rice, pandan leaves, bread flowers, meat products, bacteria and fungi, the enzymatic or biosynthetic pathway have not been revealed in details. Very few reports have illustrated the biosynthetic pathways of 2AP; however, amino acids including proline, putrescine, arginine, lysine and ornithine have been implicated as precursors for 2AP production in by different sources. The role of proline and ornithine in 2AP production was confirmed in heat treated yeast extracts (Münch et al., 1997). Romanczyk demonstrated the substantial amount of 2AP production by *Bacillus cereus* strains on medium supplemented with proline, ornithine and glutamic acid (Romanczyk et al., 1995). Costello and Henschke (2002) proved that L-ornithine increased the concentration of 2AP by 10.3-12.8 folds (258-320 µg/L). These previous reports collectively conclude that proline, ornithine, glutamic acid and putrescine are most probable precursors for 2AP biosynthesis in plants and bacterial system.

The results of Romanczyk et al (1995) had demonstrated the formation of 2AP by *B. cereus* (ATCC27552), but authors have used the thermal treatment during extraction. Also as per conclusions of previous authors there is a need of a non-thermal extraction to evaluate the process of 2AP biotransformation in bacterial system for getting the reliable results (Adams and DeKimpe 2007). Hence, a HS-SPME-GC-FID method was developed in our previous study (Deshmukh et al., 2014) which can detect and extract the 2AP at lower temperatures as compared to the methods described before.

In our previous study, several basmati rice rhizobacterial isolates producing 2AP were reported and L-ornithine (4%) was observed as the best precursor for 2AP production by rhizobacteria. The present study was based on the hypothesis that L-ornithine acts as a direct precursor of 4-aminobutanal by enzymatic or biosynthetic pathway. Present study is focused on the formation of reaction intermediates *viz.*, 4-aminobutanal and 1-pyrroline for the evaluation of mechanism of 2AP by rhizobacteria. Precursor 4-aminobutanal was used in study since 1-pyrroline is difficult to obtain in pure and stable form. 4-aminobutanal is commercially available as the diethyl acetal, from which the free aldehyde can be liberated by hydrolysis (Adams and De Kimpe 2007).

The influence of different precursors (proline, ornithine and putrescine) on the production of 2AP by different rhizobacterial strains from BM (basmati) and NBM (non-basmati) origin were also investigated to check if these rhizobacterial isolates responded in a similar way as *Bacillus* isolates isolated from cocca beans (Romanczyk et al., 1995). To elucidate specific synthesis of 2AP by rhizobacterial isolates results of the intermediate analysis are discussed to uncover mechanism followed by intermediates for 2AP biosynthesis.

II. Materials and Methods

Origin of bacterial Strains

Selection of ten bacterial strains from BM (basmati) and NBM (non-basmati) origin for precursor feeding analysis was done on the basis of screening results from our previous study **(Table 1)** (Deshmukh et al., 2015). Rhizobacterial isolates producing different amount of 2AP were selected for precursor studies.

Table. 1. 2AP production of 29 rhizobacterial isolates from basmati and non-basmati isolates.

SI.NO.	Internal number	Name of the Bacterial Isolate	Accession numbers	2AP production µg/kg (ppb)
1	**BM 2**	*Acinetobacter baylyi*	KJ372726	30.91
2	**BM 8**	*Enterobacter ludwigii*	KJ372730	54.01
3	**NBM 69**	*Staphylococcus warneri*	KJ372732	ND
4	**BM 43**	*Acinetobacter baylyi*	KJ183067	**99.06**
5	**NBM 10**	*Staphylococcus arlettae*	KJ183071	ND
6	**BM 28**	*Acinetobacter baylyi*	KJ143625	**119.3**
7	**BM 31**	*Acinetobacter baylyi*	KJ143626	**90.03**
8	**NBM 24**	*Acinetobacter junii*	KJ410756	64.80
9	**NBM 83**	*Staphylococcus Moniba*3	KJ410757	47.72
10	**BM 34**	*Acinetobacter baylyi*	KM012191	**94.34**

Bacterial culture experimental design

For each bacterial isolate, three plates were inoculated with fresh culture with and without precursors and 3 plates were left un-inoculated as control and incubated at 32 ^{o}C. Each bacterial plate was briefly removed from the incubator and subjected to HS-SPME coupled with GC-FID for analysis (Romanczyk et al., 1995).

Addition of Precursors

The production of 2AP was studied by HS-SPME-GC-MS analysis of surface cultures of different rhizobacterial isolates cultivated on PCA supplemented with L-ornithine (4%) using the optimized experimental procedure (Deshmukh et al., 2014). The response of different rhizobacterial isolates selected on the basis of 2AP producing ability was tested by inoculating them on PCA plates supplemented with L-proline (4%), L-ornithine (4%), putrescine (5%), L-arginine (1%).

Influence of 4-aminobutanal and 1-pyrroline on the production of 2AP

Being a highest 2AP producing isolate, *A. bahayai*-BM-28-KJ143625 was chosen for the further precursor studies. 4-aminobutanal diethyl acetal (0.1%, 0.2% and 0.4%) was added before or after medium sterilization (Adams and De Kimpe, 2007). PCA plates supplemented with different concentrations of 4-aminobutanal diethyl acetal and 1-pyrroline were inoculated with *A. bahayai*-BM-28. Un-inoculated controls supplemented with 4-aminobutanal diethyl acetal and 1-pyrroline was also included in the experiment.

For investigating the influence of 1-pyrroline as an immediate precursor, on the production of 2AP, 1-pyrroline (0.1%) was supplemented after medium sterilization. Un-inoculated control of 1- pyrroline (0.1 %) was also included to check the thermal formation of 2AP. 1-pyrroline is highly unstable compound. Therefore, it was synthesized from pyrrolidine, distilled and used immediately at a low concentration of 0.1%. All the rhizobacterial bacterial cultures were grown and analyzed in duplicate.

SPME-GC-MS analysis

Volatiles produced by rhizobacteria were extracted at different intervals, identified and quantified following the protocol optimized for bacterial 2AP production in our previous study (Deshmukh et al., 2014). The volatiles from the bacterial samples were identified on the basis of their mass spectra obtained using GC-MS (Shimadzu QP 5050A, Japan) with Rtx-5 capillary column (60 m x 0.25µm) and by comparing the spectra with the records of the NIST147 library and with standards of these volatiles. All the GC analysis for each sample was run in triplicate.

Synthesis of 1-pyrroline

1-pyrroline was synthesized following the method given by Adams and De Kimpe (2007). In a dried 250-ml flask, 0.25mol (33.3 g) of N-chlorosuccinimide was dissolved in 220 ml of dry diethyl ether. Equimolar amounts of pyrrolidine (17.8 g) dissolved in 10 ml of diethyl ether were carefully added, while stirring in an ice bath. The combined reagents were allowed to react for 4 h at room temperature. After filtration and evaporation to one third of the volume, two equivalents of potassium hydroxide in methanol (30 g KOH in 120 ml methanol) were added and the mixture was refluxed for 3 h. The reaction mixture was poured in 100 ml of water, extracted with diethyl ether (two times 50 ml), washed with water (100 ml) and dried (MgSO4). The obtained yield of 1-pyrroline was rather low (20%) since the evaporation of methanol involved substantial losses of volatile 1-pyrroline.

Results and discussion

Comparison of different basmati rice rhizobacterial strains:

Rhizobacterial isolates (BM and NBM origin) were cultivated on PCA plates supplemented with different precursors, in order to check if theses rhizobacterial isolates respond similarly as *Bacillus* spp. (Adams and De Kimpe 2007) using the same experimental procedure.

The production of 2AP was studied by HS-SPME-GC-MS analysis of surface cultures of different rhizobacterial isolates cultivated on PCA supplemented with 4% L-ornithine using the optimized experimental procedure **(Table 1)** (Deshmukh et al., 2014). Rhizobacterial isolates selected on the basis of 2AP producing ability were subjected to precursor feeding analysis using L-proline (4%), L-ornithine (4%), putrescine (5%), L-arginine (1%). Results for the 2AP quantification with different precursors are depicted in **Fig. 1.**

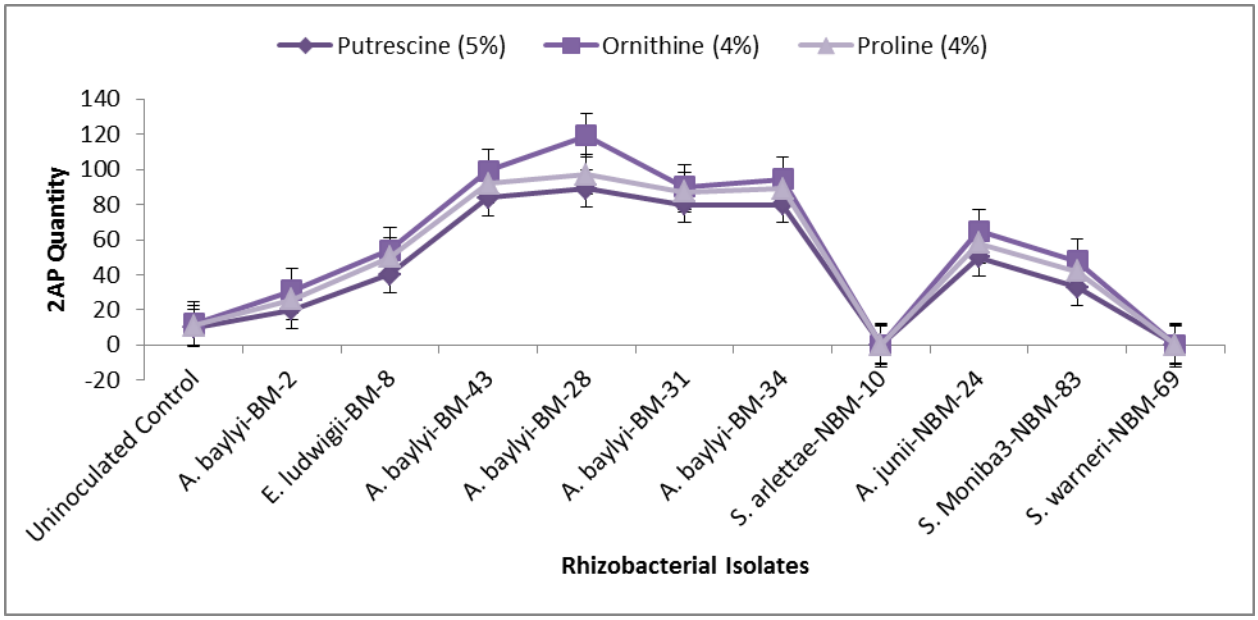

Figure 1. Quantification of 2AP using precursors at different concentrations.

Among the different precursors tested, ornithine (4%) was a good precursor for production of 2AP by different rhizobacterial isolates which was in accordance with the previous report (Deshmukh et al., 2014). Along with L-ornithine, proline and putrescine were also found as important precursors for 2AP production by rhizobacteria (**Fig. 1)**.

Previous studies with different bacterial isolates were in agreement with our study. Costello et al. (2001) suggested that in *Lactobacillus hilgardii*, Δ^1-pyrroline, a product of proline catabolism via putrescine oxidation, was the immediate precursor of the pyrroline ring of 2AP and the acetyl group was most likely formed from reaction with acetyl-CoA or acetaldehyde in either a chemical or enzymatic reaction. Additionally, detailed precursor studies have revealed that the formation of 2AP in *Bacillus cereus* proceeds via acetylation of Δ^1 pyrroline((Adams and De Kimpe 2007). In case of plant species, the biological formation of 2AP was reported from proline or ornithine in rice (Yoshihashi et al., 2015) and *Pandanus amaryllifolius* Roxb. (Thimmaraju et al., 2005). In rice, the nitrogen in the pyrroline ring of proline becomes the nitrogen in the pyrroline ring of 2AP while the carboxyl group of proline is removed and replaced with an acetyl group from another source (Yoshihashi et al., 2015). Thimmaraju et al., (2005) demonstrated that the provision of proline induces the synthesis of 2AP in *in-vitro* cultures of *Pandanus amaryllifolius.* Also, in thermal Maillard reactions, detailed precursor studies have shown that the mechanism of formation of 2AP from proline or ornithine proceeds via 1-pyrroline as the key intermediate (Hofmann and Schieberle 1998).

Influence of 4-aminobutanal diethyl acetal on the production of 2AP by *A. bahayai*-BM-28

The active degradation product of ornithine is 4-aminobutanal, which cyclizes to 1-pyrroline. 4-aminobutanal diethyl acetal is a stable form of 4-aminobutanal, from which the free aldehyde can be liberated by hydrolysis (Adams and De Kimpe 2007). Therefore, a study evaluating influence of 4-aminobutanal diethyl acetal (0.1%, 0.2% and 0.4%) on the production of 2AP by rhizobacteria isolate, *A. bahayai*-BM-28 was undertaken.

HS-SPME GC-MS analysis of the volatiles revealed that 2AP was the most important compound in the headspace profile of all cultures. A significant production of 2AP was measured in the *A. bahayai*-BM-28-KJ143625 cultures supplemented with 4-aminobutanal diethyl acetal prior to medium sterilization. No 2AP was formed in the cultures supplemented with 4-aminobutanal diethyl acetal after medium sterilization.

At 0.4 % of 4-aminobutanal diethyl acetal, an intense browning of the PCA medium was noted after sterilization and bacterial growth was inhibited which was in accordance with the previous results reported by Adams and De Kimpe (2007). The amounts of 2AP detected in the different headspace samples collected at different intervals are displayed in **Fig. 2.**

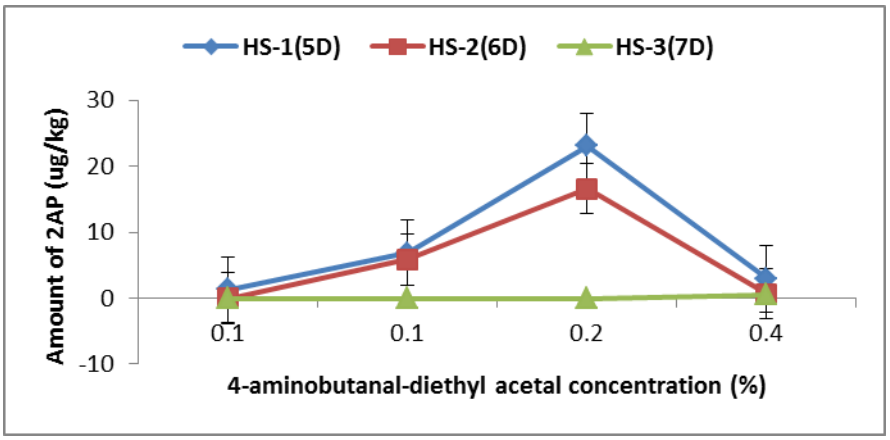

Figure 2. Quantification of 2AP using 4-aminobutanal-diethyl-acetal at different concentrations.

[Amounts of 2AP (µg/kg) detected in the headspace of un-inoculated control and *A. bahayai*-BM-28-KJ143625, inoculated PCA, supplemented with 0.1, 0.2 or 0.4 % 4-aminobutanal diethyl acetal (added prior to medium sterilization). Results of HS samples at different intervals (5-7 days).]

The results of the present study concluded that the addition of 4-aminobutanal diethyl acetal in the growth (PCA) medium before sterilization significantly increased the 2AP production by the *A. bahayai*-BM-28 cultures. The 2AP production by *A. bahayai*-BM-28 increased with the increase in concentration of 4-aminobutanal diethyl acetal up to 0.4%. The production of 2AP was the highest the first day after inoculation and decreased in later intervals. 2AP could be detected in control plates as well but in low concentration as compared to the inoculated ones.

Influence of 1-pyrroline on the production of 2AP

A. bahayai-BM-28 produced significant amount of 2AP when grown on medium supplemented with especially in the cultures supplemented with 1-pyrroline. The results of the production of 2AP are shown in **Fig. 3**. The results of the first sampling (1D) showed a significant deviation, but the second (2D) and the third headspace samples (3D) yielded very reproducible amounts of 2AP.

Very less amount of 2AP was detected in the blank media where 4-aminobutanal diethyl acetal was added after sterilization. Results clearly concluded that prior heat treatment is needed for transformation of 4-aminobutanal diethyl acetal before it can be efficiently converted to 2AP by the bacteria. As suggested by the previous studies, the heat treatment is necessary to bring about the hydrolysis of 4-aminobutanal diethyl acetal followed by the cyclization of 4-aminobutanal to 1-pyrroline. Therefore, the treatment where bacterial inoculation was with done in the medium 4-aminobutanal diethyl acetal was added after sterilization.

1-pyrroline could be detected in the un-inoculated control plates, but this 1-pyrroline could not be transformed in 2AP without bacterial cultures and therefore no 2AP was detected from these plates. The culture inoculated plates where 4-aminobutanal diethyl acetal was converted to 1-pyrroline by the process of sterilization or where 1-pyrroline was added without sterilization were detected with 2AP.

These results indicated that 2AP production occurs when 4-aminobutanal diethyl acetal deduced from ornithine/proline/putrescine is heat transformed to 1-pyrroline which ultimately is converted into 2AP by bacterial system. Similar results were observed by authors (Costello and Henschke 2002) where *Lactobacillus* sp was reported for 2AP production. The same pathway was reported by Schieberle (1990) they have demonstrated the formation of 2AP from proline and ornithine in comparable amounts by producing 1-pyrroline, an immediate precursor of 2AP. However, Citrulline and ornithine are also possible precursors of 1-pyrroline, through cyclization of 4-aminobutanal via a Strecker degradation protocol. The association of L-ornithine and L-lysine with the formation of 2AP suggests that these amino acids may be respective sources of N-heterocyclic intermediates, 1-pyrroline. An analogous pathway from ornithine, via putrescine leading to the formation of 1-pyrroline has been indicated (Fothergill and Guest 1977).

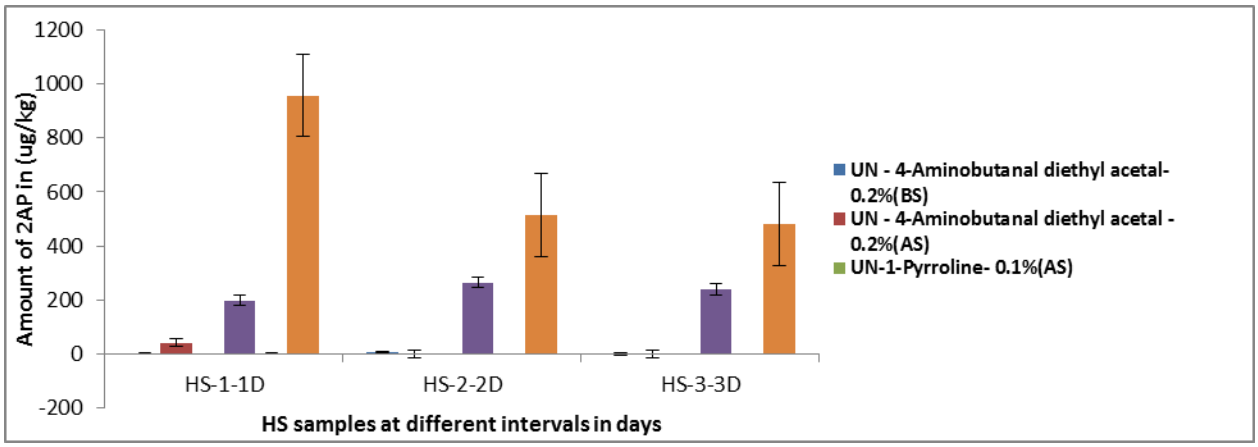

Figure 3. 2AP production (µg/kg) detected in the HS of different samples supplemented with 4-aminobutanal diethyl acetal and 1-pyrroline.

[Results of three HS samples analyzed at different intervals. Error bars represent the standard error. BS-before sterilization; AS- after sterilization; UN- uninoculated; IN-inoculated with *A. bahayai*- BM-28- KJ143625].

Enzymatic hypothesis for 2AP production

Ornithine, proline and glutamate can be converted to a common metabolite Δ^{-1} pyrroline-5-carboxylic acid, via three distinct enzymes: ornithine aminotrasferase (OAT), proline dehydrogenase (PRODH) and Δ^{-1} pyrroline-5-carboxylic acid synthetase (P5CS).

On the basis of previous finding, a biosynthetic pathway to 2AP was proposed in previous studies (Costello and Henschke 2002; Hofmann and Schieberle 1998; Huang etal., 2008, Schieberle 1990, Yoshihashi et al., 2002). The glutamic acid is converted by the bi-functional enzyme P5CS to glutamic-gamma-semialdehyde (GSA), which cyclizes spontaneously to p5CS. The latter undergoes decarboxylation from glucose with the intermediacy of fructose-1-6, diphosphate, to give 2AP. Alternatively, P5CS might directly react with MG to give final product, P5CS can also be obtained from proline by PRODH (**Fig. 4**). This pathway analysis was in accordance with the results of Huang and cowarkers. Authors concluded that BnPRODH i.e. proline dehydrogenase from *Bacillus subtilis* subsp. natto. was responsible for biosynthesis of 2AP by *B. subtilis* subsp. Natto (Huang et al., 2007).

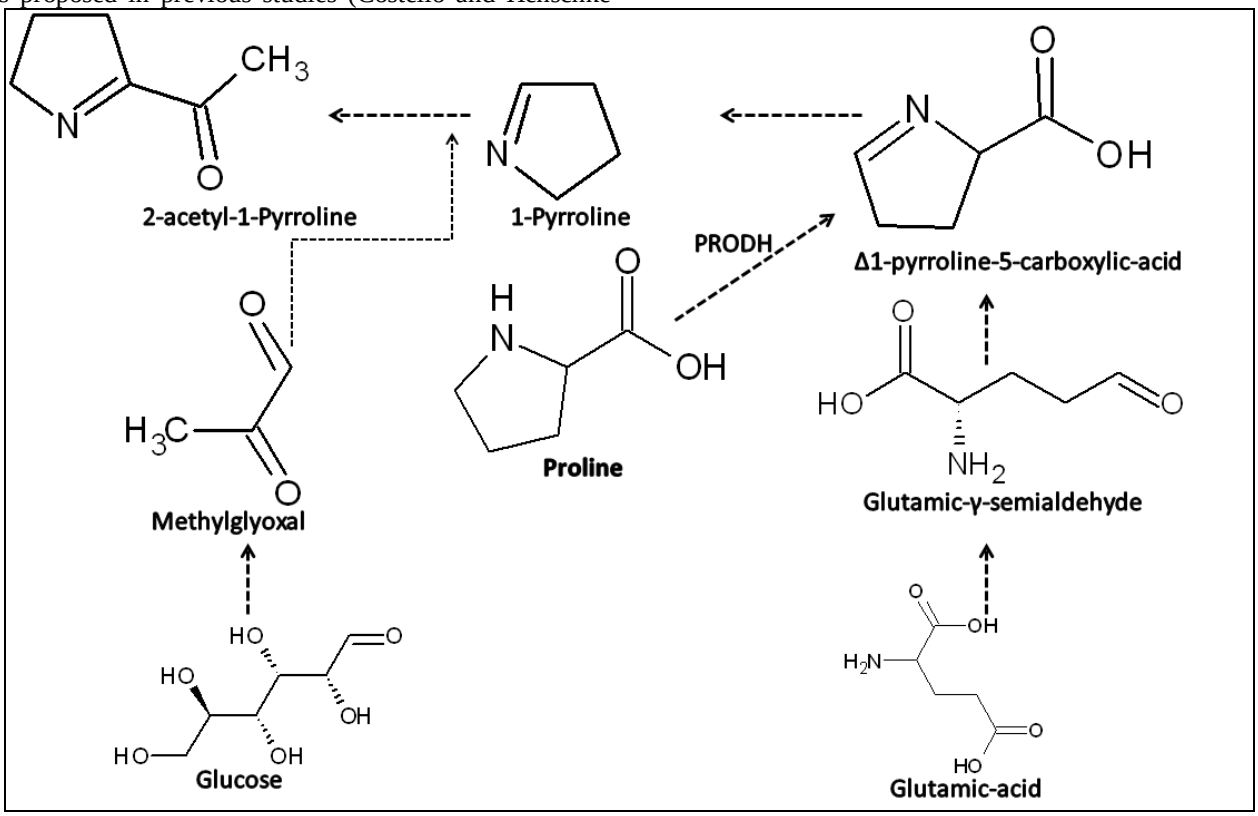

Figure 4. Pathway illustrating 2AP synthesis from proline

The pathway suggested in current study **(Fig. 5),** starts with the conversion of L-arginine to 4-aminobutyl-guanidine by arginine decarboxylase, agimate gets converted to putrescine by agimatine ureohydrolysis. Putrescine can also be formed by conversion of L-ornithine to putrescine by ornithine decarboxylase. L-ornithine involved in the reaction could be obtained from proline by transformation of intermidiates like L-1-pyrroline-5-carboxylate and L-glutamate-5-semialdehyde which finally produced L-ornithine.

In amino acid branches of this pathway, the intermidiates 1-pyrroline could accumulate from the metabolism of L-arginine and L-ornithine via the putrescine pathway. A pathway was proposed which combines the inter-conversion reactions of different amino acids and the transformation pathway of amino acids products in to 2AP by different enzymes is described in **Fig 5.** Lee and coauthors found ornithine cyclodeaminase (Ocd), as a key enzyme for ornithine biosynthesis from proline in *Corynebacterium glutamicum* (**Fig. 5**) (Lee et al., 2010). According to this pathway, proline can be used as a primary metabolite to produce ornithine directly, not via glutamate-5-semialdehyde as an intermediate. Moreover, in this pathway, proline is formed by the cyclodeamination of ornithine catalyzed by Ocd (Alam et al., 2004). In addition, Ocd under proline-supplemented conditions is a key enzyme for enhancing the biosynthesis of ornithine from proline in *C. glutamicum.* Putrescine formed in the above mentioned reactions gets converted in to 4-amino-butyraldehyde by 4-amino-butyrate aminotrasferase which exists in equilibrium with delta-1-pyrroline which is known immediate precursor for 2AP. Δ^1-pyrroline gets converted to 2AP by a bacterial system.

From the information presented, a mechanism can now be postulated for the biosynthesis of 2AP. This scheme involves the interaction of intermediates from two disparate pathways, that is i) N-heterocyclic intermediate, 1-pyrroline, derived from the catabolism of L-lysine and L-ornithine ii) bacterial conversion of accumulated 1-pyrroline.

Along with 2AP producers some non-2AP producing strains were also present in the population of rhizobacteria and this enzyme pathway provides an explanation for the inability of theses rhizobacterial strains. Absence or mutation in 4-aminobutyrate aminotransferase enzyme may render the inability of 2AP production to these rhizobacteria, which might the reason of non-production of 2AP by these bacterial isolates when grown in same conditions as that of the producers.

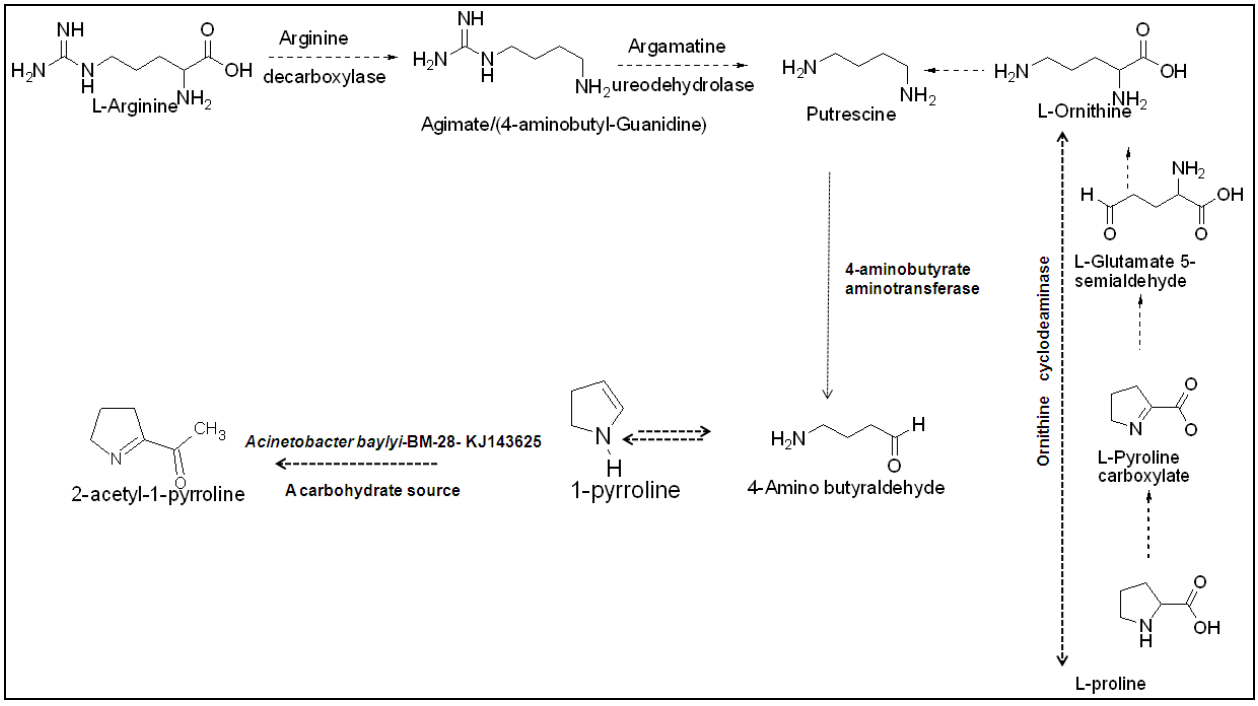

Figure 5. Proposed pathway suggesting the 2AP biosynthesis by bacterial system

III. Conclusions

The production of 2AP was enhanced by the addition of heat-treated 4-aminobutanal diethyl acetal and in particular by the addition of 1-pyrroline. This indicates that the formation of the rice flavor compound by these bacteria proceeds via the acetylation of 1-pyrroline, which is a degradation product of ornithine. Considering the results of the different studies on the thermal as well as biological origin of 2AP, a common mechanism of formation can be presumed, since in all cases the acylation of 1-pyrroline is described as the key step.

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AUTHORS

First Author – Yogita Deshmukh, Agronomy and Soil Science Division, CSIR-CIMAP, Lucknow, India
Second Author – Puja Khare, Department of Botany, University of Pune, Pune – 411007, India
Third Author – D.D. Patra, Department of Botany, University of Pune, Pune – 411007, India

Correspondence Author – E-mail:yogita.deshmukh2@gmail.com Tel Ph: 91 – 522 – 2359536, Fax: 91 – 522 – 2342666