Biotechnology Journal International

25(6): 36-47, 2021; Article no.BJI.75688
ISSN: 2456-7051
(Past name: British Biotechnology Journal, Past ISSN: 2231–2927, NLM ID: 101616695)

Screening of Yeasts Other than Saccharomyces for Amino acid Decarboxylation

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJI/2021/v25i630157

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/75688

Original Research Article

Received 09 September 2021
Accepted 05 November 2021
Published 27 December 2021

ABSTRACT

This study is aimed at screening non-Saccharomyces for amino acids decarboxylation potentials. The yeasts were isolated from banana fruit and honey purchased from markets in Rivers State. The isolation and molecular identification of yeast isolates were according to standard microbiological procedures. A plate assay method for amino acid decarboxylation (biogenic amine production) screening was used. Wild Non-Saccharomyces yeast (NSY) were identified as *Candida tropicalis* Pe 1 (B7), *Candida tropicalis* WC65-1 (B10), *Candida tropicalis* WC57 (H4), *Clavispora lusitaniae* WM03 (H7), and a Commercial Wine yeast (CY) identified as *Candida tropicalis* zhuan4 (CY). The NSYs and CY were biogenic amine producers, from L-histidine and glutamic acid; strain variability from glycine, proline, glutamine, and asparagine decarboxylation; while L-arginine, lysine, tyrosine, cysteine, leucine, and phenylalanine were not decarboxylated at a concentration of 0.1 %. The increase in amino acid concentration influenced the number of amino acids decarboxylated - phenylalanine and leucine; L-histidine, glycine, asparagine and glutamic acid were decarboxylated by wild NSY and CY, while the strain variability of phenylalanine, proline, leucine and glutamic acid decarboxylation. The amino acids L-arginine, lysine, tyrosine, and cysteine were not decarboxylated. In terms of the concentration of amino acids, L-histidine and glutamic acid were

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decarboxylated and arginine, lysine, tyrosine, and cysteine were not decarboxylated by wild NSY isolates and CY. The Chi-square Test and Kendall's Test of concordance suggest that there is no association between the amino acid concentrations (0.1 and 1 %) and biogenic amine production (P-value > 0.05). The wild NSY and CY are biogenic amine-producers, and the increase in amino acid concentration influences biogenic amine production concerning some amino acids.

Keywords: Non-Saccharomyces; Amino acids decarboxylation; Biogenic amine; Candida tropicalis

1. INTRODUCTION

Non-Saccharomyces are ascomycetous yeasts like *Candida*, *Kloeckera*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Pichia*, *Metschnikowia*, *Schizosaccharomyces*, *Saccharomycodes*, *Torulaspora*, *Issatchenkia*, *Metschnikowia*, *Kluyveromyces* and *Rhodotorula* that expresses enzymes of technological importance or mycotoxins [1-5] are involved in the fermentation of most indigenous foods and alcoholic beverages [6-14]. Presently, non-Saccharomyces genera like *Kloeckera* sp., *Candida* sp., *Torulaspora delbrueckii*, *Pichia* sp, *Metschnikowia* sp., and *Hanseniaspora* sp. have been employed by biotechnologist in the production of fermented foods, and beverages [15-17] due to low concentrations of glycerol, acetaldehyde, acetic acid and ethyl acetate production [18].

Biogenic amines (BAs) are low molecular weight non-volatile nitrogenous organic bases compounds possessing biological activity formed by microorganisms (bacteria and fungi) through decarboxylation (the elimination of the carboxyl group with the formation of corresponding products of amine and carbon (iv) oxide) from free amino acid in food by decarboxylases, [19-22] and they are used as an indicator of toxified foodstuff [23-24] because if ingested at high concentration induces foodborne intoxications, causing undesirable physiological effects in sensitive humans [25] as they are associated with products that involves a ripening or fermentation [26].

Free Amino Acids (FAAs) could either be the essential or non-essential amino acids, which are not part of proteins or peptides: threonine, glutamic acid, glycine, alanine, b-alanine, tryptophan, 1-methylhistidine, proline, arginine, glycine, alanine, histidine, glutamic acid, and anserine [27]. This kind and amount of FAAs impacts the organoleptic properties of fermented food and biogenic amines precursor. Thus, biogenic amines are associated with variety of raw, processed, and fermented foods, rich in protein (FAAs), decarboxylated by microorganisms [28-31] and the decarboxylation of amino acid into corresponding biogenic amine is strain dependent [32-36,22].

It had been reported of biogenic amine producing Lactic Acid Bacteria (LAB) such as Leuconostoc strains an intensive tyramine former, and *Latobacillus buchneri* associated with putrescine formation, but *Oenococcus oeni* strains and some commercial starter bacteria have been identified as non-producer of histamine, tyramine and putrescine [23]. *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, *Pediococcus*, *Oenococcus oeni* isolated during fermentation produced biogenic amine [37-38], *Enterococcus sp*. and coliform bacteria isolated from Dutch-type semi-hard cheese expressed functional genes for biogenic amine [39] and *Enterobacter aerogenes* also produced large amounts of BA in fish [40] while *Clostridium* and *Pseudomonas* strains in traditional soybean pastes are BA producers [41].

The strains of yeast species, *Kloeckera apiculata*, *Brettanomyces bruxellensis*, *Metschnikowia pulcherrima*, Candida stellata, and *Saccharomyces cerevisiae* have been reported to exhibit amino acid decarboxylation [42].*Trichosporon asahii*, *Rhodotorula mucilaginosa*, *Candida rugosa*, *Yarrowia lipolytica*, *Pichia jadinii* and *Debaryomyces hansenii* and the genera Candida have been reported to produce different BA in different environment [43]. This study is aimed at screening of non-Saccharomyces for amino acid decarboxylation potential, using twelve amino acids for the screening exercise

2. MATERIALS AND METHODS

2.1 Collection of Samples

Yeast isolates were obtained from honey and wholesome ripe banana fruit. The wild honey was purchased from Ekpoma, Edo State, Nigeria, while, wholesome ripe banana fruit was bought from Fruit Garden Market, Port Harcourt, Rivers State, Nigeria. The samples were

transferred to Microbiology Laboratory, Department of Microbiology of Rivers State University. The ripe banana was washed with clean water to remove dirt after which it was peeled for further analysis and CY GV1 imported from the US.

2.2 Isolation of Yeasts from Samples

Ten grams (10g) of the ripe banana fruit and 10 ml of the wild honey was transferred aseptically into 250ml conical flasks containing 90ml sterile peptone broth. Both broths were incubated for 24-48 hours at 30 ℃ for further analysis. Aliquot (0.1ml) of the broth was transferred into prepared Yeast Extract Peptone Dextrose (YEPD) agar plates (1% yeast extract, 2% peptone, 2% dextrose) supplemented with chloramphenicol (0.003g/L) and was spread evenly using a sterile bent glass rod. Plates were incubated at 30 ℃ for 48 hours (Hong and Park, 2013). After incubation, plates were observed for growth and were sub-cultured on YEPDA plates. The morphology of the yeasts was confirmed through macroscopic (appearance on YEPDA plates) and microscopically by viewing under the light microscope at X100 magnification (Ali and Latif, 2016) after staining. Both the wild yeast strains and the commercial wine yeasts were further identified molecularly (PCR and sequencing of the ribosomal ITS region). The commercial wine (CY) yeast GV1 was used as a reference to the isolated wild yeasts.

2.3 Molecular Characterization

The molecular characterization was carried out in the Bioinformatics Service Laboratory, Ibadan, Nigeria. The CTAB method as described by (Ali and Latif, 2016) was used in extracting DNA from yeast strains. In this method, 24 hours yeast cultures in YEPD broth were centrifuged at maximum speed. About 10mg of yeast cells for each strain were taken and pre-warmed in 200 µl of solution I at 65°C containing 1.4M NaCl, 2% CTAB, 20mM EDTA (pH 8.0), 0.2% β-mercaptoethanol and 100mM Tris-HCl (pH 8.0) was introduced, mixed well, and incubated at 65°C for 15-20 minutes in the water bath. After incubation, all tubes were cooled for 3-5 minutes and the same volume of solution II (Chloroform: Isoamyl alcohol, 24:1) was added, mixed thoroughly, and centrifuged at 14,000 rpm for 10 minutes at room temperature. The aqueous phase (upper) was taken from each Eppendorf separately and 3M Na acetate (1/10) was introduced in each Eppendorf along with an equal volume of cold isopropanol or double volume of cold absolute ethanol, mixed it gently, and placed on ice for 10 minutes. All tubes after incubation were centrifuged at 12000 rpm at 4°C for 15 minutes and the supernatant was disposed. About 500µl of chilled 70% ethanol (solution III) was added directly for washing pellet and then centrifuged at 14000 at 4°C for 2 minutes. The pellet was air-dried after discarding supernatant from each tube. The pellet was resuspended in 50µl double deionized water or TE-buffer and stored at −20°C. The yield of DNA was quantified by Spectrophotometer. The ribosomal DNA internal transcribed spacer region: ITS1 (GTAGGTGAACCTGCGG) and ITS4 (TCC GCTTATTGATATGC) was used to amplify the DNA [44]. The reaction mixture contained 100ng DNA, 5µl of 10pmol each oligonucleotide primer, 3µl of 25mM MgCl2, 3µl of 250mM dNTPs mixture, and Taq DNA polymerase (5units) in a total volume of 50 µl. PCR conditions were as follows: 3 min. at 94 °C followed by 35 cycles (45 sec at 94°C, 45 sec. at 55°C (annealing temperature), 1 min. at 72°C, and final extension for seven min. at 72°C. The amplified product was determined by running on 0.8% agarose gel and visualized using a UV illuminator and photographed. More so, PCR products of the partially amplified-ITS region were subjected to restriction fragment length polymorphism (RFLP) for two restriction endonucleases TaqI and HaeIII. The reaction mixture contained 3.0 µl of 1X buffer (R-buffer for BsuRI (HaeIII) and unique-buffer for TaqI), 15.0 µl PCR products (approximately 1.0 µg), 1µl of specific endonuclease, and 11µl of deionized water with a total volume of 30µl. The reaction mixtures were incubated at their specific temperatures as recommended by the manufacturer's instructions (Fermentas) The restriction fragments were separated along with a DNA 100bp ladder on 1.5% w/v agarose gel and photographed after visualization under UV light. Finally, 2.5µl of the purified PCR products were sequenced using the Applied Biosystems ABI PRISMTM 3100 DNA sequence Analyzers with the BigDye® Terminator v3.1 Cycle Sequencing kit and protocols [45]. The obtained DNA sequence was blasted on the NCBI gene bank to confirm the identities of the various yeasts.

2.4 Amino Acids Decarboxylation Assay

Screening of yeast isolates for amino acid decarboxylation potential was carried out by a plate assay method. The amino acids L-histidine, tyrosine, phenylalanine, glutamine, lysine, leucine, glycine, cystine, proline, asparagine, glutamic acid, and L- arginine were

utilized. Briefly, ten microliters of a saturated yeast culture were applied onto Yeast Extract Peptone Dextrose (YEPD) agar plates, containing 0.1 % and 1 % (w/v) of each chosen amino acid and 0.006% (w/v) bromocresol. After incubation at ambient for 5-7 days, the plates were analyzed for the presence/absence of a purple around the yeast colony: amino acid decarboxylation was considered positive when there a purple colour surrounding the yeast colony [46] modified. CY GV1 was concerned as a yardstick to judge the wild yeast isolates.

3. RESULTS

The four wild NSY isolates isolated from banana fruit (B7 and B10), wild honey (H4 and H7) and one Commercial Wine Yeast GV1 (CY) were identified as *Candida tropicalis* Pe 1 (B7), *Candida tropicalis* WC65-1 (B10), *Candida tropicalis* WC57 (H4), *Clavispora lusitaniae* WM03 (H7) and *Candida tropicalis* zhuan4 (CY) as shown in Table 1 and genomic DNA fingerprinting in Fig. 1.

Table 1. Sequence identification from National Center for Biotechnology Information (NCBI) blastN hits and their Percentage Relatedness

Isolate code	NCBI BLASTN relative	Accession number	E Value	Percentage (%) relatedness
B7	*Candida tropicalis* Pe 1	MK752669	2.50E-179	93.10
B10	*Candida tropicalis* WC65-1	EF190223	0	95.00
CY	*Candida tropicalis* zhuan4	EF192229	0	92.10
H4	*Candida tropicalis* WC57	EF198007	0	94.80
H7	*Clavispora lusitaniae* WM03	KF268353	8.52E-35	77.60

Key: B = yeast isolated from banana, H= yeast isolated from honey, CY= commercial wine yeast

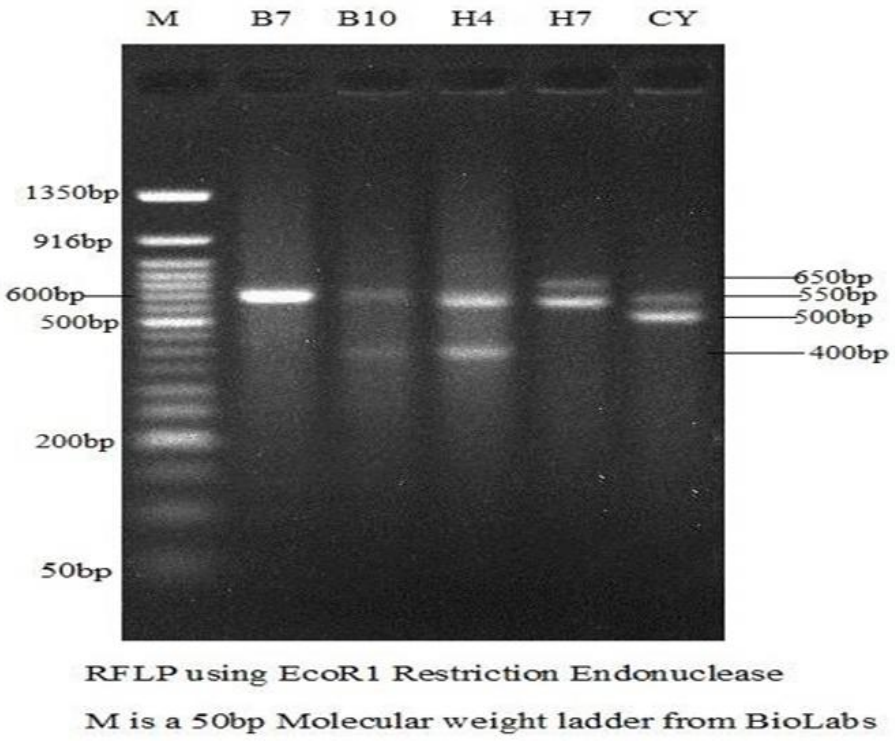


Fig. 1. DNA fingerprinting employing Restriction Fragment Length Polymorphism (RFLP) method and EcoR1 restriction endonuclease for cutting the DNA sequence of yeast isolates

The results of biogenic amine production potential of NSY isolates on plate agar containing 0.1 % of amino acids are demonstrated in Table 2., as biogenic amine positive agar plates turn purple and negative agar plates retain the initial pink colour. All the NSYs and CY were biogenic amine producers, from L-histidine and glutamic acid; strain variability was shown in biogenic amine production from glycine, proline, glutamine, and asparagine, while L- arginine, lysine, tyrosine, cysteine, leucine, and phenylalanine were not decarboxylated.

Down the table of Table 2, *Candida tropicalis* Pe 1 (B7) synthesized biogenic amine from L-histidine, glycine, proline, glutamine, and glutamic acid; *Candida tropicalis* WC65-1 (B10) from L-histidine, glycine, glutamine, asparagine, and glutamic acid; *Candida tropicalis* WC57 (H4) from L-histidine, glycine, proline, asparagine, and glutamic acid; *Clavispora lusitaniae* WM03 (H7) from L-histidine and glutamic acid; and *Candida tropicalis* zhuan4 (CY) from L-histidine, glutamine, asparagine, and glutamic acid.

Table 2. Pattern of biogenic amine production by NSY isolates from 0.1% of amino acid

Amino acids	B7	B10	H4	H7	CY
L - Arginine	-	-	-	-	-
L - Histidine	+	+	+	+	+
Glycine	+	+	+	-	-
Lysine	-	-	-	-	-
Tyrosine	-	-	-	-	-
Cysteine	-	-	-	-	-
Phenylalanine	-	-	-	-	-
Proline	+	-	+	-	-
Leucine	-	-	-	-	-
Glutamine	+	+	-	-	+
Asparagine	-	+	+	-	+
Glutamic acid	+	+	+	+	+

+ = biogenic amine production; - = no biogenic amine production

The results of biogenic amine production potential of wild NSY isolates and CY with increase in amino acid concentration, from 0.1 - 1 (%) are demonstrated in Table 3., which reveal increase in the number of amino acids decarboxylated by each of the yeasts. It was shown that phenylalanine and leucine were included among the decarboxylated amino acids; L-histidine, glycine, asparagine, and glutamic acid were decarboxylated by both wild NSY and CY, while strain variability was shown in biogenic amine production from phenylalanine, proline, leucine, and glutamic acid. Amino acids L-arginine, lysine, tyrosine, and cysteine were not decarboxylated by NSY isolates and CY.

Table 3 shows that *Candida tropicalis* Pe 1 (B7) and *Candida tropicalis* WC65-1 (B10) decarboxylated L-histidine, glycine, proline, leucine, glutamine, asparagine, glutamic acid; decarboxylated L- histidine, glycine, glutamine, asparagine, and glutamic acid; *Candida tropicalis* WC57 (H4) and *Clavispora lusitaniae* WM03 (H7) decarboxylated L- histidine, glycine, phenylalanine, leucine, asparagine, glutamic acid, but differ in glutamine decarboxylation, and *Candida tropicalis* zhuan4 (CY) decarboxylated L- histidine, glycine, phenylalanine, glutamine, asparagine, and glutamic acid.

Table 3. Pattern of biogenic amine production by NSY from 1% Amino acids

Amino acids	B7	B10	H4	H7	CY
L - Histidine	+	+	+	+	+
L - Arginine	-	-	-	-	-
Glycine	+	+	+	+	+
Lysine	-	-	-	-	-
Tyrosine	-	-	-	-	-
Cysteine	-	-	-	-	-
Phenylalanine	-	-	+	+	+
Proline	+	+	+	+	-
Leucine	+	+	+	+	-
Glutamine	+	+	+	-	+
Asparagine	+	+	+	+	+
Glutamic acid	+	+	+	+	+

+ = biogenic amine production; - = no biogenic amine production

Comparing the pattern of biogenic amine production among the amino acids on Table 2 and Table 3., showed that L-histidine and glutamic acid were decarboxylated, and arginine, lysine, tyrosine, and cysteine were not decarboxylated by wild NSY isolates and CY with increase in amino acid concentration, but increase in amino acid concentration resulted in decarboxylation of the following amino acids, leucine and asparagine by *Candida tropicalis* Pe 1 (B7); proline and leucine by *Candida tropicalis* WC65-1 (B10); phenylalanine, leucine and glutamine by *Candida tropicalis* WC57 (H4); phenylalanine, glycine, proline, leucine, asparagine, and glycine and phenylalanine by CY.

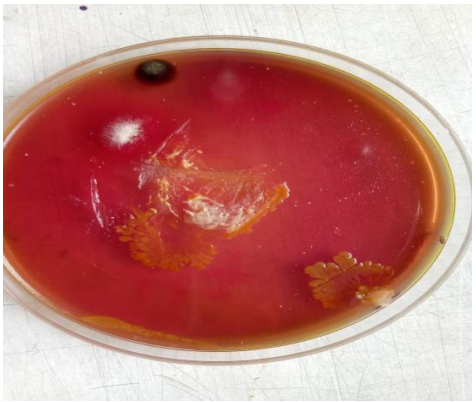
The comparison and relationship between yeast isolates and amino acid concentrations about biogenic amine production is represented in table 4; using statistical tools such as Chi square,

Correlation coefficient, Cramer's measure of association and Test of concordance. The Chi square Test depict that there is no association in their pattern of biogenic amine production at P-value > 0.05, table shown evidence indicates that there is a positively linear but weak relationship in their pattern of biogenic amine production at spearman's rho = 0.108, and Test of concordance shows that there is discordant in pattern of biogenic amine production regarding amino acid concentration at P-value > 0.05 (Table 4). The degree of association is regarded as not generally useful at Cramer's V- square < 0.1.

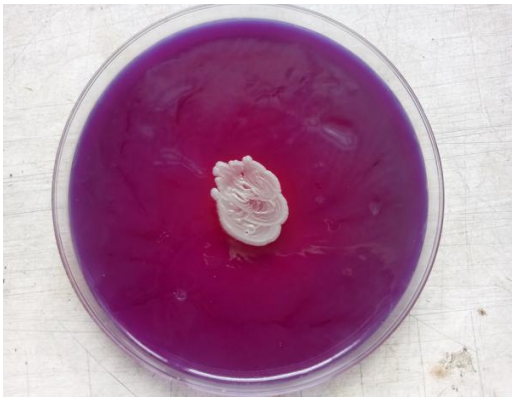
The comparison and relationship between amino acid concentrations have been stated explicitly in table 5, using statistical tools like Chi square, Correlation coefficient, Cramer's measure of association and Test of concordance. The Chi square Test and Kendall's Test of concordance suggests that there is no association between the amino acid concentrations (0.1 and 1 %) about biogenic amine production at P-value > 0.05 for *Candida tropicalis* Pe 1 (B7), *Candida tropicalis* WC65-1 (B10), *Candida tropicalis* WC57 (H4), and *Candida tropicalis* zhuan4 (CY), but for *Clavispora lusitaniae* WM03 (H7) there is association at P-value < 0.05. The correlation coefficient test indicate that there is a positive linear, but relatively weak relationship between amino acid concentrations and biogenic amine production by *Candida tropicalis* Pe 1 (B7), *Candida tropicalis* WC65-1 (B10), and *Candida tropicalis* zhuan4 (CY); positive linear, and moderate relationship between amino acid concentrations and biogenic amine production by *Candida tropicalis* WC57 (H4), and positive linear, and relatively strong relationship between amino acid concentrations and biogenic amine production by *Clavispora lusitaniae* WM03 (H7). Cramer's measure of association indicates that *Clavispora lusitaniae* WM03 (H7) had weak degree of association at spearman's rho of 0.185, while the other yeasts degree of association is regarded as not generally useful at Cramer's V- square < 0.1.

4. DISCUSSION

Biogenic amine positive agar plates turn purple and negative agar plates retain pink colour is a qualitative method of screening microorganisms for the ability of biogenic amine production – amino acid decarboxylation [46]. It was observed that non-Saccharomyces yeasts both wild yeast isolates and Commercial wine yeast demonstrated the potential of biogenic amine production in the presence of FAAs. The study agrees with the report of that *Saccharomyces cerevisiae*, *Kloeckera apiculata*, *Candida stellata*, *Metschnikowia pulcherrima* and *Brettanomyces bruxellensis* as biogenic amine-producers [47] which is the responsibility of the enzymes and amino acid-decarboxylases [48]. Although, microorganisms generally possess the potential for decarboxylation of amino acids which results to biogenic amine production [49] like LAB that is implicated with the risk of biogenic amine formation [20,39,33,38,23,50]. The studies reported by Bäumlisberger [51] Landete *et al*. [25] and Wu *et al* [52] stated contrary views to the report of isolating non-biogenic amine producing yeasts and biogenic amine degrading yeasts isolates.



a. Biogenic amine negative plate of Czapek Dox Agar culture



b. Biogenic amine positive plate of Czapek Dox Agar culture

Fig. 2. Yeast culture on Czapek Dox Agar for biogenic amine screening.

Table 4. Comparison and relationship between yeast isolates and amino acid concentrations for biogenic amine production

Yeast isolate	0.1% Amino acid	1% Amino acid	Chi Square	Correlation coefficient	Cramer's measure of association	Test of concordance
B10	5(23.81%)	7(20%)	P-value = 0.893	Spearman's rho = 0.108	Cramer's V-square = 0.019	P-value = 0.208
B7	5(23.81%)	7(20%)				
CY	4(19.05%)	6(17.14%)				
H4	5(23.81%)	8(22.86%)				
H7	2(9.52%)	7(20%)				

Table 5. Comparison and relationship between amino acid concentrations and biogenic amine production

Yeast isolate	Amino acid Concentration	BA negative	BA positive	Chi Square (P-value)	Correlation coefficient (Spearmans rho)	Cramer's measure of association (Cramer's V-square)	Test of concordance (P-value)
B10	0.1%	7(58.33)	5(41.67%)	0.41	0.166	0.027	0.207
	1%	5(41.67%)	7(58.33)				
B7	0.1%	7(58.33)	5(41.67%)	0.41	0.166	0.027	0.207
	1%	5(41.67%)	7(58.33)				
CY	0.1%	8(66.67%)	4(33.33%)	0.408	0.169	0.028	0.203
	1%	6(50%)	6(50%)				
H4	0.1%	7(58.33%)	5(41.67%)	0.219	0.250	0.062	0.109
	1%	4(33.33%)	8(66.67%)				
H7	0.1%	10(83.33%)	2(16.67%)	0.035	0.430	0.185	0.017
	1%	5(41.67%)	7(58.33%)				

The potential to form biogenic amine from L-histidine, glycine, and glutamine was associated with and are common among the yeasts screened for biogenic amine formation; why there were differential decarboxylation of the other amino acids among the yeasts, which demonstrate the variation among the species. This study collaborate with the report of Tristezza *et al*. [46] and Beneduce *et al*. [32] that yeast isolates are able to decarboxylate histidine and histamine as one of the main biogenic amine associated with wine. Landete [25] and Moon *et al*. [41] stated that amino acid-decarboxylases are not broadly distributed among microbes, due to variability in microbial cells. Thus, the ability of microbial decarboxylation of amino acids is highly variable: as species of many genera are proficient of decarboxylating one or more amino acids, as demonstrated by histamine-producing *Clostridium* strain, and tyramine-producing *Pseudomonas* strain isolated from the same source. The variability in amino acid decarboxylation is a function of the presence or absence of decarboxylase genes, which reveal the correlation between genotypic detection and phenotypic expression [39,33].

The increase in the concentration of amino acid from 0.1 % to 1 % led to increase in the number of amino acids that was decarboxylated for biogenic amine production by the wild yeast isolates and the commercial wine yeast; statistically, showing positive linear, and relatively strong or weak relationship between amino acid concentrations and biogenic amine production. This study agrees with a previous study by Özdestan and Üren [53] who state that obtainability of FAAs contributes to the presence and accumulation of biogenic amines in foods. Then, inferring low concentrations of biogenic amines in ciders correlates to low contents of the corresponding precursor amino acids [31]. It reported also that some forms of treatment with time enhances increase in the biogenic amine production [40,50], which could have resulted due to change in pH of the environment [32]. it is very hard to find wines without any biogenic amine, because the major biogenic amines' production are influenced by a number of oenological factors [54-55].

5. CONCLUSION

The wild yeast isolates and the Commercial wine yeast screened for biogenic amine production were all biogenic amine producers, possessing high affinity for amino acid histidine and glutamine as they were decarboxylated even at 0.1 %, while amino acids like arginine, lysine, tyrosine, and cysteine are not decarboxylated by the yeasts, which could be due to lack of the specific decarboxylase genes required. There is no association between change in amino acid concentration and biogenic amine formation in the course of yeasts utilizing the various amino acid; rather, there is variability in biogenic amine production with respect to change in amino acid concentration and individual amino acid respectively.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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