



Evaluation of biogenic amines producing potential of bacteria associated with proteinous food

Felicia Oyinkansola Akinwande and Simiat Olanike Jimoh*

Industrial Microbiology Laboratory, Microbiology Programme, Department of Biological Sciences, College of Natural and Applied Sciences, Fountain University Osogbo, Osun State, Nigeria.
Email: olanike771@gmail.com

Received 4 January 2019; Received in revised form 14 June 2019 ; Accepted 30 July 2019

ABSTRACT

Aim: The aim of this study was to assay for the biogenic amine-producing capacity of bacteria isolated from proteinous food.

Methodology and results: Previously characterized bacterial isolates (*Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*) obtained from proteinous food samples (smoked fish and yoghurt) were subjected to proteolytic analysis using nutrient agar supplemented with 0.2 g/mL casein and decarboxylase activity using nutrient broth supplemented with 0.004 g/mL amino acids (histidine, tyrosine, asparagines, leucine and lysine). Isolates that expressed proteolytic and decarboxylase activities were screened for biogenic amine producing capacity using decarboxylase broth which was supplemented with an amino acid (tyrosine). Biogenic amines obtained in this research were classified into primary amine and secondary amine based on their qualitative characteristics. Confirmatory and quantitative analysis of biogenic amines produced was done using high-performance liquid chromatography. The confirmatory screening revealed the presence of methylamine, ethylamine, putrescine, cadaverine, histamine, spermidine, phernylethylamine, spermine, agmatine, tyramine, dopamine, tryptamine, norepinephrine and serotonin respectively. Total biogenic amines produced by *S. aureus* was 70.12 mg/kg, *K. pneumoniae* (62.58 mg/kg) and *E. coli* (56.57 mg/kg) respectively.

Conclusion, significance and impact of study: Enzymatic decarboxylation of free amino acids and other metabolic processes by the test organisms (*S. aureus*, *E. coli* and *K. pneumoniae*) leads to production of biogenic amines which can be used as a quality indicator in food in terms of degree of spoilage, use of non-hygienic raw material and poor manufacturing environment. Thus, effect of biogenic amines obtained in this research would be determined by individual toxicological threshold which can be extremely variable from few mg/kg in sensitive person to several hundred mg/kg in healthy person. The concentrations of each biogenic amine quantified are within the limit but their toxic effects depend on the type of amine, the presence of modulating compounds and the efficiency of an individual's detoxification mechanism.

Keywords: Decarboxylase, proteolytic activity, spermidine, histamine, *Staphylococcus aureus*, primary amines

INTRODUCTION

Biogenic amines (BAs) are natural bases, which can be available in nourishment and can cause several unfavorable responses in the consumers. BAs are produced by microorganisms (bacteria and fungi) through the activity of decarboxylases, which act specifically on certain amino acids through the elimination of the carboxyl group with the development of corresponding amine and carbondioxide (Marcobal *et al.*, 2012; Qi *et al.*, 2014). Proteinous food provides a sufficient medium for microbial growth because of its rich and balanced chemical composition thus microbial development and resulting in situ generation of metabolites with putative

toxicological impacts is supported by the restricted inhibitory activity of naturally occurring antimicrobial substances in dairy products (Claeys *et al.*, 2013).

Biogenic amine produced by Lactic acid bacteria has been depicted as a trademark procured through horizontal gene transfer linked to plasmids. In specific cases, this attribute can be considered as species-trademark, for example, the production of tyramine in *Enterococcus* or putrescine in *Lactococcus* (Ladero *et al.*, 2012). In correlation to their concentration and toxicological impacts, the most vital BAs in nourishments are histamine (heterocyclic amine obtained from histidine), tyramine and 2-phenylethylamine (obtained from aromatic amino acids; tyrosine and phenylalanine

*Corresponding author

respectively), tryptamine (heterocyclic biogenic amine from tryptophan), putrescine (polyamine obtained through direct decarboxylation of ornithine, which occurs after the decarboxylation of arginine to agmatine), and cadaverine (a polyamine derived from lysine) (Wunderlichová *et al.*, 2014).

MATERIALS AND METHODS

Assay for decarboxylase activity of biogenic amine-producers

Previously characterized organisms namely *S. aureus*, *E. coli* and *K. pneumoniae* isolated from yogurt and smoked fish samples obtained from hawkers within Osogbo metropolis, Osun state, Nigeria were subcultured on decarboxylase agar which composed of tryptone (0.5 g), yeast extract (0.5 g), sodium chloride (0.5 g), glucose (0.1 g), tween 80 (0.1 g), magnesium sulphate (0.02 g), manganese sulphate (0.005 g), iron sulphate (0.004 g), calcium carbonate (0.01 g), amino acid (2.0 g), bromothymol blue (0.006 g), bacteriological agar (2 g) per 100 mL of distilled water at pH 5. Colour change in bromothymol blue indicator from yellow to blue indicated presence of decarboxylase enzyme (Da Silva *et al.*, 2002).

Assay for proteolytic activity of biogenic amine-producers

Biogenic amine-producers which exhibited decarboxylase activities were subjected to proteolytic activities according to the methodology of Ouoba *et al.* (2003) using nutrient agar supplemented with 0.2 g/mL of casein and incubated at 37 °C for 24 h. Zones of clearance around the isolates indicated production of proteolytic enzyme.

Preliminary screening of biogenic amine-producers

Biogenic amine producers were screened according to the methodology of Da Silva *et al.* (2002) with slight modification. Isolates with decarboxylase and proteolytic activities were subcultured in nutrient broth supplemented with 0.004 g/mL amino acids (either histidine, tyrosine, lysine, leucin, or asparagine) independently and incubated at 30 °C for 48 h. After incubation period, a loopful of each culture medium was inoculated separately on decarboxylase agar at 30 °C for 48 h utilizing spread plate technique. Presence of blue radiance on agar plate was interpreted as positive for amine production. Thus, culture medium containing amino acid with the widest blue radiance was selected for biogenic amines production.

Assay for biogenic amine production

Biogenic amine-producing bacteria that showed positive results were inoculated into amine-production medium containing decarboxylase broth [tryptone (0.5 g), yeast extract (0.5 g), sodium chloride (0.5 g), glucose (0.1 g),

tween 80 (0.1 g), magnesium sulphate (0.02 g), manganese sulphate (0.005 g), iron sulphate (0.004 g), calcium carbonate (0.01 g), bromothymol blue (0.006 g)] supplemented with tyrosine (2 g) per 100 mL of distilled water at pH 5 separately (Joosten *et al.*, 1989).

Extraction of biogenic amine from production medium

Positive culture broth (0.2 mL) was centrifuged for 10 min in 10 mL of 0.4 M perchloric acid at 3000 rpm to acquire cell-free supernatant; thus aggregate volume of the supernatant was adjusted to 26 mL adding 0.4 M of perchloric acid. Furthermore, 200 µL of 2 M sodium hydroxide, saturated sodium carbonate (300 µL) and 2 mL of dansyl chloride were added and incubated at 40 °C for 45 min. After incubation period, ammonium carbonate (100 µL) was added, left for 30 min and the volume was adjusted to 5 mL with acetonitrile. The reaction mixture was centrifuged for 5 min at 2500 rpm and supernatant was recovered by filtering using 0.45 µm syringe filter (Joong-Seok *et al.*, 2004).

Qualitative analysis of biogenic amine produced

Supernatant (1 mL each) obtained above was mixed with 2 mL of 2.0 M hydrochloric acid; cooled to 5 °C in an ice bath and 5 drops of frosty 20% aqueous sodium nitrite solution was added and the mixture obtained were tested for presence of primary aliphatic amine, primary aromatic amine, secondary amine, and tertiary amine according to the methodology of Linares *et al.* (2010). The immediate evolution of a colourless gas (nitrogen) indicates presences of primary aliphatic amine. Absence of gas production but separation of an insoluble yellow or orange liquid from the solution indicated presence of a secondary aliphatic or aromatic amine. Absence of gas production and no separation of yellow liquid from the solution (i.e no reaction occurred) indicated presence of tertiary amine. Addition of few drops of cold reaction mixture to a cold solution of 50 mg beta-naphthol in 2 mL of 2 M sodium hydroxide leads to formation of an orange/red azo dye and diazonium salts which indicates presence of primary aromatic amine.

Quantitative analysis of biogenic amine produced

Biogenic amines produced were analyzed using High-performance liquid chromatography (HPLC) as depicted by Magwamba *et al.* (2010). One milliliter of 2 M sodium hydroxide was added to 2 mL of supernatant followed by 1 mL of benzoyl chloride (2%). The reaction mixture was vortex for 1 min and stored at 25 °C for 40 min. The reaction was stopped by adding 2 mL of saturated sodium chloride solution, and the solution was extracted twice with 2 mL of diethyl ether. The upper organic layer was transferred into a clean tube after mixing and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 500 mL of acetonitrile and 20 mL aliquots were injected into the HPLC. The mobile phase consisted

of acetonitrile of HPLC grade (eluant A) and ultrapure water (eluant B) which were filtered and degassed before use. Chromatographic separation utilized an isocratic elution method with acetonitrile and ultrapure water in the ratio 60:40 respectively. The flow rate was 1.2 mL/min for 6.5 min to ensure complete separation and the derivatized amines were detected with a UV detector at 254 nm. Biogenic amine standards such as methylamine, ethylamine, putrescine, cadaverine, histamine, spermidine, phernylethylamine, spermine, agmatine, tyramine, dopamine, tryptamine, norepinephrine and serotonin were analyzed together with test samples. During analysis, a standard solution was also injected intermittently between test samples to check chromatographic consistency. Each sample was injected twice and the peak heights of the biogenic amine standard solutions were used to prepare standard curves and to further determine the amine concentrations in the test samples.

RESULTS

Biogenic amine-producers decarboxylase and proteolytic activity

Decarboxylating activity of the test organisms (*S. aureus*, *E. coli* and *K. pneumoniae*) using different amino acids are as shown on Table 1. Zones of blue colouration around the isolates indicated presence of decarboxylase enzyme required for amine production. Among the amino acids utilized, decarboxylase agar containing tyrosine as the main protein source had the widest zone of blue colouration of 34±0.23 mm compared to histidine (21±0.18 mm), leucine (21±0.16 mm), asparagine (18±0.04 mm), and lysine (17±0.11 mm). Thus, tyrosine was selected for qualitative and quantitative analysis. Furthermore, zones of clearance around the isolates grown on casein agar indicated production of proteolytic enzyme (Table 2). *Staphylococcus aureus* produced the widest zone of clearance of 36±0.23 mm.

Table 1: Decarboxylating activity of bacterial isolates using different amino acids.

Amino acids	Decarboxylating activity of bacterial isolates		
	<i>K. pneumoniae</i>	<i>S. aureus</i>	<i>E. coli</i>
Histidine	16±0.12	21±0.18	8±0.04
Tyrosine	22±0.19	34±0.23	18±0.12
Asparagine	12±0.08	18±0.14	12±0.14
Leucine	11±0.13	21±0.16	10±0.15
Lysine	13±0.15	17±0.11	11±0.12

Data represented Mean ± SEM of triplicate analysis

Table 2: Proteolytic activity of bacterial isolates on casein agar.

Isolates	Proteolytic activity [Zones of clearance (mm)]
<i>K. pneumoniae</i>	11± 0.17
<i>S. aureus</i>	36±0.23
<i>E. coli</i>	9±0.15

Data represented Mean ± SEM of triplicate analysis

Qualitative analysis of biogenic amine

Biogenic amines such as primary aromatic amine and secondary amine are identified based on their chemical reaction. All test organisms produced secondary aromatic amines due to absence of gas production but separation of an insoluble yellow liquid from solution while *S. aureus* and *E. coli* produced primary aromatic amine due to formation diazonium salts which are stable at low temperatures (Table 4). *Staphylococcus aureus* and *E. coli* produced both primary aromatic amines and secondary amine while *K. pneumoniae* produced secondary amine only.

Table 3: Preliminary screening of biogenic amine producers.

Amino acids	Presences of blue radiance		
	<i>K. pneumoniae</i>	<i>S. aureus</i>	<i>E. coli</i>
Histidine	+	++	+
Tyrosine	++	+++	+
Asparagine	+	++	+
Leucine	+	++	+
Lysine	+	++	+

+ : slight blue radiance, biogenic amine present
 ++ : wide blue radiance, biogenic amine present
 +++ : widest blue radiance, biogenic amine present

Quantitative analysis of biogenic amines produced

Biogenic amines concentration obtained using HPLC are as shown on Tables 5-7. The confirmatory screening revealed the presence of methylamine, ethylamine, putrescine, cadaverine, histamine, spermidine, phernylethylamine, spermine, agmatine, tyramine, dopamine, tryptamine, norepinephrine and serotonin. Total biogenic produced by *S. aureus*, *K. pneumoniae* and *E. coli* were 70.12 mg/kg, 62.58 mg/kg and 56.57 mg/kg, respectively.

DISCUSSION

Production of biogenic amines (BAs) by microorganisms isolated from yogurt and smoked fish can be used as quality index which are expressed as chemical indicators of the hygienic conditions of raw material. BAs are produced through different pathways depending on the producer bacterium, genes/enzymes it possesses, and the ecological niche from which it originates (Nannelli *et al.*, 2008). Biogenic amines such as methylamine, ethylamine, putrescine, cadaverine, histamine,

Table 4: Qualitative analysis of biogenic amines produced.

Test Organism	Histidine				Tyrosine				Asparagine				Leucine				Lysine			
	PL	PR	S	T	PL	PR	S	T	PL	PR	S	T	PL	PR	S	T	PL	PR	S	T
<i>S. aureus</i>	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	-	+	-
<i>E. coli</i>	-	+	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	+	+	-
<i>K. pneumonia</i>	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-

Results represent triplicate analysis. PL, Primary aliphatic amine; PR, Primary aromatic amine; S, Secondary amine; T, Tertiary amine; +, Biogenic amine produced; -, No production of biogenic amine

spermidine, phernylethylamine, spermine, agmatine, tyramine, dopamine, tryptamine, norepinephrine and serotonin obtained in this research through microbial decarboxylation of amino acids revealed that the test organisms (*S. aureus*, *E. coli* and *K. pneumoniae*) are decarboxylase producing species thus their existence in food samples can eventually lead to decarboxylation of Proteinous food during aging and storage (Stadnik and Dolatowski, 2010). Production of histamine, tyramine, cadaverine, putrescine, tryptamine and phenylethylamine by test organisms isolated from yoghurt indicate failure in hygienic quality of milk used for yoghurt production or during fermentation process (Pintadoa *et al.*, 2008).

Table 5: Quantification of biogenic amine produced by *S. aureus*.

RetTime [min]	Amount [mg/kg]	Biogenic amine
7.651	4.87×10^{-3}	Methylamine
8.654	3.22×10^{-4}	Ethylamine
9.688	4.47	Putrescine
11.366	6.31	Cadaverine
12.829	18.45	Histamine
14.157	27.93	Spermidine
14.875	5.10×10^{-2}	Phernylethylamine
15.388	11.59	Spermine
16.053	1.15×10^{-2}	Agmatine
16.545	1.23	Tyramine
17.761	1.11×10^{-3}	Dopamine
18.588	1.21×10^{-4}	Tryptamine
18.772	4.63×10^{-2}	Norepinephrine
19.346	1.86×10^{-2}	Serotonin
Total	70.12	

Production of these BAs in the cytoplasm occurred through a One-step decarboxylation reaction, followed by their excretion outside the cell, which requires systems for active transport and amino acid decarboxylase enzymes. Variation in the decarboxylase activities of the test organisms was dependent on the ability of the organism to transport precursor amino acids (Table 1) into the cytoplasm through an antiporter protein in exchange for the resulting BA. Although tyrosine medium with the widest zones of blue radiance requires a uniporter transport system using TyrP protein than the antiporter system (Table 3) (Marcobal *et al.*, 2012). Proteolysis is the most important phenomenon during fermentation process. In this research, casein is being ruptured by

proteolytic enzymes released by the test organisms thus causes increase in free amino acids which are subjected to decarboxylation reactions catalyzed by specific bacterial decarboxylase activity resulting in formation of amines (Table 2).

Table 6: Quantification of biogenic amine produced by *E. coli*.

RetTime [min]	Amount [mg/kg]	Biogenic amine
7.648	6.80×10^{-3}	Methylamine
8.628	8.90×10^{-4}	Ethylamine
9.685	8.65	Putrescine
11.363	6.67	Cadaverine
12.826	3.94	Histamine
14.153	23.88	Spermidine
14.878	2.49×10^{-2}	Phernylethylamine
15.384	12.14	Spermine
16.062	5.35×10^{-3}	Agmatine
16.541	1.22	Tyramine
17.700	2.18×10^{-3}	Dopamine
18.616	4.86×10^{-5}	Tryptamine
18.765	1.33×10^{-2}	Norepinephrine
19.302	4.64×10^{-3}	Serotonin
Total	56.57	

Table 7: Quantification of biogenic amine produced by *K. pneumonia*.

RetTime [min]	Amount [mg/kg]	Biogenic amine
7.698	8.61×10^{-4}	Methylamine
8.716	4.21×10^{-3}	Ethylamine
9.782	4.85	Putrescine
11.340	7.67	Cadaverine
12.797	21.52	Histamine
14.160	24.31	Spermidine
14.881	3.67×10^{-2}	Phernylethylamine
15.394	3.16	Spermine
15.989	2.09×10^{-2}	Agmatine
16.555	1.01	Tyramine
17.789	7.11×10^{-4}	Dopamine
18.533	2.46×10^{-4}	Tryptamine
18.805	1.08×10^{-3}	Norepinephrine
19.301	6.11×10^{-5}	Serotonin
Total	62.58	

Variation in the total biogenic amine concentration (Tables 5-7) produced by each test organism is dependent on factors such as specific bacterial strains present, level of decarboxylase activity and availability of amino acid (Rivas *et al.*, 2010). Putrescine is one of the most abundant and frequently found polyamines in dairy products but low yield of putrescine (4.47-8.65 mg/kg) with corresponding high yield of spermidine (23.88-27.93 mg/kg) and spermine (3.16-12.14 mg/kg) obtained in this research showed the ability of the test organisms to convert putrescine to spermidine and spermine through condensation reactions catalyzed by spermidine synthase and spermine synthase respectively (Shah and Swiatlo, 2008). Despite their cellular functions, excess levels of spermine and spermidine can lead to toxicity which could decrease blood pressure, inhibit blood clotting and provoke respiratory symptoms and neurotoxicity resulting in renal insufficiency (Pegg, 2013).

Food intoxication which occurred from consumption of histamine causes symptoms such as flushing of the face, neck and upper arms, oral numbness and/or burning, metallic taste, headache, itchy rash, heart palpitations, asthma attacks, hives, gastrointestinal symptoms, and difficulties in swallowing (Knöpe *et al.*, 2014). Thus, histamine yield (3.94-21.52 mg/kg) produced by the test organisms utilized in this research can serve as a yardstick to assess the hygienic quality of their origin (yoghurt and fish). Production of tyramine by the test organisms also revealed the tendency of the organisms to cause food intoxication when grown in proteinous food because tyramine has a vasoconstrictor effect on human thus causes dietary-induced migraine, increased cardiac output, nausea, vomiting, respiratory disorders, and elevated blood glucose (Marcobal *et al.*, 2012). Increase in blood pressure due to tyramine concentration causes heart failure or brain hemorrhage (Naila *et al.*, 2010). Presences of tyramine also increases adherence of *E. coli* O157:H7 to intestinal mucosa thus having a detrimental effect on the gut microbiota (Russo *et al.*, 2012).

Ability of the test organisms to produce such biogenic amines in amino acid precursor medium indicated that their presence in proteinous food can constitute a risk to the consumer health because ingestion of food containing high amounts of BAs is implicated in various pharmacological and toxicological reactions such as headaches, heart palpitations, vomiting, and diarrhoea (Özogul *et al.*, 2011). Under normal conditions in humans, exogenous amines ingested with food are rapidly detoxified by monoamine oxidase (MAO) and diamine oxidase enzymes but the severity of BA toxicological effects depends on the intake with food, individual allergy, consumption of MAO inhibiting drugs, alcohol and other food amines (Sathyanarayana and Yeragani, 2010).

CONCLUSION

Enzymatic decarboxylation of free amino acids and other metabolic processes by *S. aureus*, *E. coli* and *K. pneumoniae* leads to production of biogenic amines which can be serve as a quality indicator in food in terms of degree of spoilage, use of non-hygienic raw material and poor manufacturing environment. Although, intake of exogenous polyamines are beneficial in the treatment of some geriatric diseases and prolonging human life through consumption of food polyamines that are abundant in the Mediterranean diet (Binh *et al.*, 2010) and utilization of polyamine pathway as a rational target for chemoprevention and chemotherapeutics (Nowotarski *et al.*, 2013). Thus, effect of biogenic amines obtained in this research would be determined by individual toxicological threshold which can be extremely variable from few mg/kg in sensitive person to several hundred mg/kg in healthy person (Hungerford, 2010). The concentrations of each biogenic amine quantified are within the limit but their toxic effects depend on the type of amine, the presence of modulating compounds and the efficiency of an individual's detoxification mechanism (Rauscher-Gaberng *et al.*, 2009).

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