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Original article

Tyramine-producing enterococci are equally detected on tyramine production medium, by quantification of tyramine by HPLC, or by *tdc* gene-targeted PCR

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Abstract – The presence of biogenic amines (BAs) in foods is not acceptable as the consumption of food and beverages containing high levels of BAs could result in toxicological effects for human health. BAs are mainly produced by the microbial decarboxylation of certain amino acids. Among the various BAs, tyramine is the most commonly cited and abundant BA produced by several bacterial genera. Various biochemical, chromatographic and molecular methods are used for the detection of BAs. Among different lactic acid bacteria, enterococci have been found to be the most abundant tyramine producers. In the present study, 28 previously isolated bacteriocinogenic strains of *Enterococcus* sp. were tested for their ability to produce tyramine by qualitative, quantitative and molecular methods. Correlations between these methods were also investigated. A total of 19 enterococcal strains were found to produce tyramine by all the methods used. A low level of correlation was found between the results of two different decarboxylating media used. "Improved medium" detected more tyrosine-positive colonies than "tyrosine production medium". However, a 100% correlation was observed between the results observed with "tyrosine production medium", *tdc* gene-targeted polymerase chain reaction and tyramine quantification by high performance liquid chromatography. Therefore, these methods may be used to complement each other in the detection of tyramine-producing enterococci in foods.

food safety / biogenic amine / tyramine / enterococci / HPLC / PCR

摘要 – 酪胺产生培养基、HPLC 定量分析酪胺和 *tdc* 靶基因 PCR 法检测产酪胺肠球菌。 基于食品安全性，在食品和饮料中生物胺的含量有限量标准。 生物胺的形成是在微生物的参与发生的氨基酸脱羧反应的产物。 酪胺是最典型和含量最高，其形成与微生物有关的生物胺。 关于生物胺的分析方法有生物化学法、色谱法和分子生物学法。 在乳酸菌中，肠球菌是大量酪胺的制造者。 本研究采用定性、定量和分子生物学方法对已经分离的 28 株产细菌素的肠球菌的产酪胺量进行了分析，测定结果证明 19 株肠球菌能产生酪胺，而且这些方法之间具有较好的相关性。 但在使用了两种脱羧基的培养基时，各种方法检测结果的相关性较低。 "改进的培养基"检测到的产酪氨酸的阳性菌株数目高于

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“酪氨酸产生培养基”中检测的数目。然而，“酪氨酸产生培养基”法、*tdc* 靶基因 PCR 法和 HPLC 酪胺定量分析法，这三种方法分析结果的相关性为 100%。因此，这些方法相互补充，可以用来分析食品中产酪胺的肠球菌。

食品安全 / 生物胺 / 酪胺 / 肠球菌 / HPLC / PCR

Résumé – Les entérocoques producteurs de tyramine sont détectés de façon équivalente sur le milieu « tyramine production medium », par quantification de la tyramine par HPLC, et par PCR ciblant le gène *tdc*. La présence d’amines biogènes (AB) dans les aliments n’est pas acceptable parce que la consommation d’aliments ou boissons contenant des teneurs élevées en AB pourrait avoir des effets toxicologiques sur la santé humaine. Les AB sont principalement issues de la décarboxylation de certains acides aminés par les micro-organismes. Parmi les différentes AB, la tyramine est l’AB la plus fréquemment détectée et la plus abondante, et est produite par plusieurs genres bactériens. Différentes méthodes biochimiques, chromatographiques et moléculaires sont utilisées pour la détection d’AB. Parmi différentes bactéries lactiques, les entérocoques s’avèrent être les plus producteurs de tyramine. Dans la présente étude, 28 souches d’*Enterococcus* bactériocinogènes isolées antérieurement ont été testées pour leur capacité à produire de la tyramine par des méthodes qualitatives, quantitatives et moléculaires. La corrélation entre ces méthodes a été également étudiée. Parmi ces souches d’entérocoques, 19 se sont révélées positives par toutes les méthodes utilisées. Un faible niveau de corrélation était trouvé entre les résultats de deux milieux gélosés testés. Le milieu « improved medium » détectait plus de colonies positives que le milieu « tyrosine production medium ». Cependant, une corrélation à 100 % était observée entre les résultats obtenus sur le milieu « tyrosine production medium », par quantification de la tyramine par HPLC et la détection de gène *tdc* par PCR. Ces méthodes peuvent donc être utilisées en complément l’une de l’autre pour détecter les entérocoques producteurs de tyramine dans les aliments.

sécurité alimentaire / amine biogène / tyramine / entérocoques / HPLC / PCR

1. INTRODUCTION

Recent trends in food safety are directed toward an increasing search for trace compounds that adversely affect human health. The presence of biogenic amines (BAs) in foods is of public concern for the food industry and the regulatory agencies [13]. BAs represent a group of low molecular weight basic nitrogenous compounds that are mainly produced by the microbial decarboxylation of certain amino acids [14]. BAs, however, may be of endogenous origin at very low concentration in non-fermented foods such as fruits, vegetables, meat, milk and fish. Unfortunately, all fermented foods (dairy products, beer, wine, sausages, etc.) carry the risk of high concentration of BAs as a result of growth of contaminating or indigenous microflora exhibiting amino acid decarboxylase activity [28].

Histamine, tyramine, putrescine, cadaverine and phenylethylamine are the most common BAs that are found in fermented foods [14] and are mainly produced by lactic acid bacteria (LAB) [19]. Their occurrence and accumulation is influenced by the environmental factors (temperature, pH, availability of free amino acid, etc.) that affect the growth and decarboxylase activity of these organisms [9, 27]. High amounts of these BAs may negatively alter the organoleptic properties of the contaminated products, and consumption of these foods could have several toxicological effects such as respiratory distress, headache, hyper- or hypotension and allergies [14, 27]. These problems are particularly more severe in sensitive consumers having low levels of monoamine and diamino-oxidase enzymes (belonging to the BA detoxification system) [3]. Moreover, alcohol ingestion increases the undesirable effects that

are produced due to the presence of BAs [22]. *cis*-Urocanic acid has been recognized as a mast cell degranulator and showed synergistic effect with endogenous histamine in spoiled fish [17]. More specifically, tyramine is the most studied BA and is known to cause headaches, low blood pressure, hypertension, edema, vomiting, diarrhea, etc. [14, 27]. Although there are no regulations governing the BA content in most foodstuffs, the Nutritional codex of the Slovak Republic had determined the maximal tolerable limit for the tyramine in cheese (200 $mg \cdot kg^{-1}$) [13]. The presence of BA in the milk is quite low, about 1 $mg \cdot mL^{-1}$, but in the cheese their content reaches about 1052 $mg \cdot kg^{-1}$ [10].

The genus *Enterococcus* like other LAB forms an important part of food and is known to have an ambiguous relationship to human nutrition. They play a significant role in the development of typical taste, flavor and aroma of several fermented foods. Furthermore, the production of bacteriocins by enterococci (enterocins) is well documented [11]. These technological applications have led us to propose enterococci as adjunct starters or protective cultures in fermented foods. Moreover, enterococci are nowadays promoted as probiotics that have several beneficial health claims [1, 11]. However, unlike most of LAB, *Enterococcus* species are not having a "generally recognized as safe" (GRAS, USA) or "qualified presumption of safety" (QPS, Europe) status. This is because some of the enterococcal strains are typical opportunistic pathogens that cause disease especially in the nosocomial settings, which may in part be linked to the presence of antibiotic resistance and virulence determinants [23]. Furthermore, it has also been observed that the prolific growth of enterococci in food products leads to the formation of significant levels of tyramine [10, 18, 19].

Methods that can rapidly detect BAs producing strains in foodstuffs are required if food quality and safety is to be assured. This can help the food industry to inspect raw materials destined for use in food production [9]. Specific differential culture media for the presumptive identification of BA-producer bacteria have been developed [6, 26]. Analytical chromatographic methods used for routine BA analysis of food substrates have also been applied to bacterial cultures [20, 24]. Recently, several polymerase chain reaction (PCR)-based methods have been developed targeting specifically the amino acid decarboxylase gene. These molecular methods, in addition to their rapidity and specificity, offer the advantage of the identification of producer bacteria before the amine is synthesized [15].

As there are few reports on the relative comparison among these methods of analysis of BAs produced by enterococci, the present study aims at the evaluation of the 28 strains of *Enterococcus* sp. that were isolated in a previous study [12] for tyramine production by qualitative (decarboxylating medium), quantitative (high performance liquid chromatography, HPLC) and molecular methods (detection of *tdc* gene by PCR).

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

The 28 enterococcal strains used in this study are listed in Table I. *Enterococus faecalis* NCDC 114 and *Lactococcus lactis* subsp. *cremoris* NCDC 61 used as positive and negative control, respectively, were procured from the National Collection of Dairy Cultures (NCDC) and National Dairy Research Institute, Karnal, India. All strains were grown in deMan, Rogosa and Sharpe (MRS) medium that was purchased from HiMedia Laboratories, Mumbai, India and incubated under aerobic conditions in a shaker incubator (Innova42, New Brunswick

Table I. Detection of tyramine produced by enterococcal strains by different methods.

S. No.	Strain	Tyrosine decarboxylation		Tyrosine decarboxylase gene	Tyramine quantification by HPLC ($mg \cdot L^{-1}$)	
		Improved medium	TPM		Aerobic condition	Anaerobic condition
1.	*E. faecalis* KH 12	+	+	+	7382	6723
2.	*E. faecium* KH 24	+	−	−	−	−
3.	*E. faecium* DH 28	−	+	+	7618	5079
4.	*E. faecium* RH 31	+	+	+	718	512
5.	*E. faecium* RH 33	+	+	+	9063	8234
6.	*E. faecium* RH 38	+	+	+	8456	7510
7.	*E. faecium* DH 56	−	+	+	7841	7051
8.	*E. faecium* KH 58	−	−	−	−	−
9.	*E. faecium* DH 59	+	+	+	5693	3871
10.	*E. faecium* CH 60	+	+	+	7866	5938
11.	*E. faecalis* KH 62	+	+	+	4322	3568
12.	*E. faecalis* KH 67	+	+	+	8197	7587
13.	*E. faecalis* KH 70	−	−	−	−	−
14.	*E. faecalis* KH 72	+	+	+	2947	1845
15.	*E. faecium* RH 78	+	+	+	4091	3407
16.	*E. faecium* KH 79	−	−	−	−	−
17.	*E. faecium* KH 81	+	+	+	1567	943
18.	*E. faecalis* KH 91	+	+	+	1317	1139
19.	*E. faecalis* KH 93	+	+	+	3184	2868
20.	*E. faecalis* KH 98	+	−	−	−	−
21.	*E. faecium* FH 99	+	−	−	−	−
22.	*E. faecium* FH 102	+	+	+	717	475
23.	*E. faecium* RH 106	−	+	+	4731	3370
24.	*E. faecium* KH 110	+	+	+	5389	4620
25.	*E. faecalis* KH 111	+	−	−	−	−
26.	*E. faecium* DH 115	+	−	−	−	−
27.	*E. faecium* KH 126	+	+	+	4913	3173
28.	*E. faecium* FH 133	+	−	−	−	−
29.	*E. faecalis* NCDC 114	+	+	+	8063	7708
30.	*L. lactis* subsp. *cremoris* NCDC 61	−	−	−	−	−

TPM: tyramine production medium.

Scientific, New Jersey, USA) at 70 rpm at 37 °C until mid-log phase.

2.2. Molecular characterization of *Enterococcus* sp.

All 28 biochemically characterized enterococcal isolates [12] were subjected to molecular characterization by the PCR method for definite confirmation of species. The genomic DNA from all the cultures was extracted by the method described by Pospiech and Newmann [25]. Primer pairs EM1A (5′-TTGAGGCAGACCAGATTGACG-3′)/EM1B (5′-TATGACAGCGACTCCGATTCC-3′) [4] targeted ATP-dependent

DNA helicase RecG gene and E1 (5′-ATC AAGTACAGTTAGTTCT-3′)/E2 (5′-ACG ATTCAAAGCTAACTG-3′) [8] for D-ala: D-ala ligase (*ddl*) gene was used for the characterization of *E. faecium* and *E. faecalis*, respectively. The amplification program used was as follows: 95 °C for 5 min, then by 35 cycles at 95 °C for 45 s, 51 °C for 45 s and 45 °C for 1 min for *E. faecium* and *E. faecalis*, respectively, and 72 °C for 1 min and a final extension at 72 °C for 10 min in a thermocycler, EP Gradient (Eppendorf Mastercycler, Hamburg, Germany). The integrity of PCR products was assayed by the development of single bands following electrophoresis for 1 h at 100 V in 1.5% (w/v) agarose gels in Tris-EDTA buffer. *E. faecium* DSM 900 that was kindly provided by Ulrich Schillinger (Institute of Microbiology and Toxicology, Federal Research Centre for Nutrition, Karlsruhe, Germany) and *E. faecalis* ATCC 29212 that was kindly provided by Annalisa Serio (Dipartimento di Scienze degli Alimenti, Universita degli Studi di Teramo, Via C.R. Lerici, Mosciano Stazione TE, Italy) were used as reference strains in the PCR-based identification.

2.3. Qualitative estimation of tyramine-forming ability

The primary screening and qualitative estimation of tyrosine decarboxylation ability of the 28 enterococcal strains were done by the two different decarboxylating media, the improved medium [2], based on the color change of indicator and the modified tyramine production medium (TPM) [16]. All the enterococcal strains along with both positive and negative cultures were subcultured 5–10 times in an MRS broth containing 0.1% tyrosine and 0.005% pyridoxal-5-phosphate to promote enzyme induction before the actual screening test. After that, the strains were inoculated in the improved broth medium as well as streaked on TPM plates simultaneously and incubated for four days at 37 °C under both aerobic and anaerobic conditions in a gas jar (GasPak™ 100 system, complete, BBL Systems, USA). Strains were considered tyrosine decarboxylase positive if the color of the indicator turned yellow to violet in improved medium and a clear zone due to solubilization of tyrosine around the colonies in the case of TPM.

2.4. Detection of the tyrosine decarboxylase gene

The *tdc* gene detection was performed using a duplex PCR method, with two sets of primers, TD2/TD5 [7], targeted *tdc* gene and BSF8/BSR1541, 16S rRNA universal primers, as PCR internal control [30] (both sets of primers synthesized by Imperial Life Science, Gurgaon, India). The PCR mixture consisted of 1 ng of total DNA, primer concentrations 20 pmol for TD2 and TD5 and 5 pmol for BSF8 and BSR1541, 1 U of Taq DNA polymerase, 5 μL of 10 X Taq buffer and 1 μL dNTPs (50 pmol) in a final volume of 50 μL. The amplification program was as follows: 95 °C for 5 min, then by 35 cycles at 95 °C for 45 s, 52 °C for 45 s and 72 °C for 1 min and a final extension at 72 °C for 5 min in thermocycler, EP Gradient (Eppendorf Mastercycler, Hamburg, Germany). The presence of PCR products was assayed by electrophoresis as described in Section 2.2.

2.5. Quantitative estimation of tyramine by HPLC

Tyramine concentration produced by enterococcal strains was quantified by HPLC. All tested strains were inoculated in a TPM broth that was supplemented with 10 $g \cdot L^{-1}$ of tyrosine and incubated simultaneously under both aerobic and anaerobic (in GasPak™ 100 system, complete, BBL systems, USA) conditions at 37 °C for three days in a shaker incubator to see the effect of the presence and the absence of oxygen.

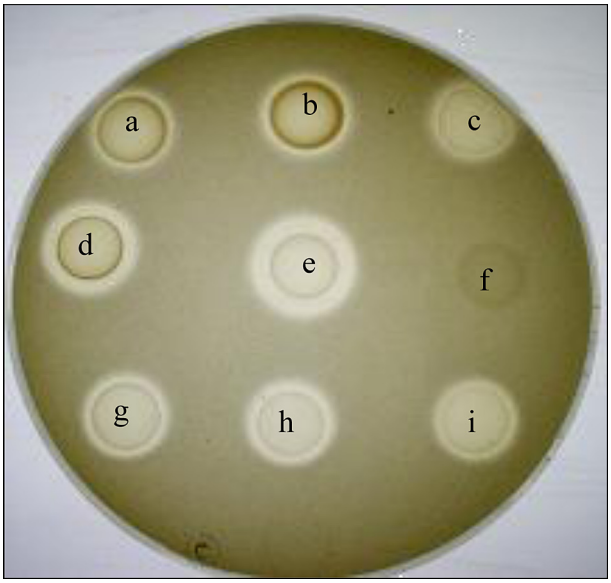

Figure 1. Tyrosine production medium (TPM) to detect tyrosine decarboxylating enterococcal strains. a, *E. faecalis* KH 12; b, *E. faecium* DH 28; c, *E. faecium* RH 31; d, *E. faecium* RH 33; e, *E. faecalis* NCDC 114 (positive control); f, *L. lactis* subsp. *cremoris* NCDC 61 (negative control); g, *E. faecium* RH 38; h, *E. faecalis* KH 62 and i, *E. faecalis* KH 67.

Sample preparation involved centrifugation of the broth, filtration through a 0.22 µm filter and derivatized with orthophthalaldehyde. Finally, the sample was injected into the port of the HPLC system (Shimadzu Corporation, Kyoto, Japan), equipped with a UV detector and an intelligent pump. HPLC separation was performed on a C_{18} (2) column (250 × 4.60 mm, 100 Å, particle diameter 5 µm) that was purchased from Phenomenex (Macclesfield, Cheshire, UK). A gradient elution was used comprising two buffer systems, viz: (i) 0.1 mol·L^{-1} ammonium acetate buffer, pH 7.3 (buffer A) and acetonitrile (buffer B). The eluant gradient began with 45% 0.1 mol·L^{-1} ammonium acetate and 55% acetonitrile and ended with 10% 0.1 mol·L^{-1} ammonium acetate and 90% acetonitrile for 11 min at 40 °C oven temperature with the flow rate of 1 mL·min^{-1}. The detection was done at the wavelength of 254 nm. Standard tyramine was purchased from HiMedia Laboratories, Mumbai, India. Regression coefficient (r) of peak area against tyramine concentration was calculated.

3. RESULTS

3.1. Screening for tyrosine decarboxylase activity

Preliminary screening of the tyrosine decarboxylase activity among the 28 strains of *Enterococcus* was done using the two different media, improved medium and TPM. Of the 28 strains, 22 (78%) showed positive results in improved medium indicated by a color change of medium [2]; while only 19 strains (68%) produced a clear zone (Fig. 1) on TPM indicating tyrosine decarboxylase activity [16] (Tab. I). However, with improved medium three strains (DH 28, DH 56 and RH 106) showed negative, while six strains (KH 24,

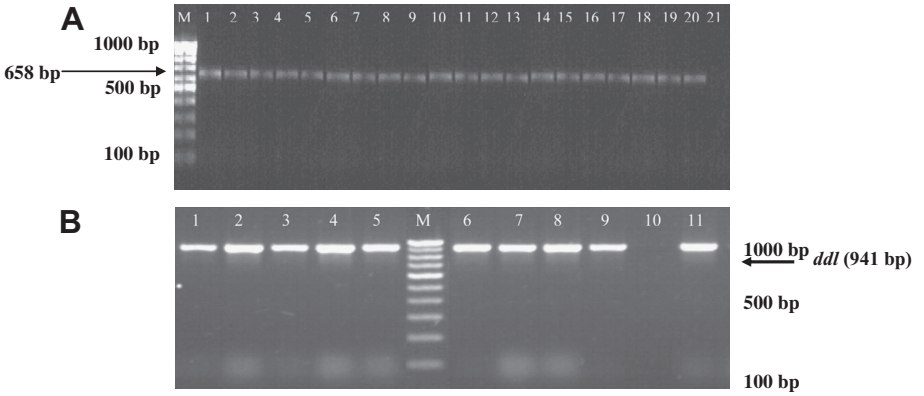

Figure 2. Molecular identification of *Enterococcus* sp. (A) *E. faecium* 1, *E. faecium* BFE 900 (positive control); 2, *E. faecium* KH 24; 3, *E. faecium* DH 28; 4, *E. faecium* RH 31; 5, *E. faecium* RH 33; 6, *E. faecium* RH 38; 7, *E. faecium* DH 56; 8, *E. faecium* KH 58; 9, *E. faecium* DH 59; 10, *E. faecium* CH 60; 11, *E. faecium* RH 78; 12, *E. faecium* KH 79; 13, *E. faecium* KH 81; 14, *E. faecium* FH 99; 15, *E. faecium* FH 102; 16, *E. faecium* RH 106; 17, *E. faecium* KH 110; 18, *E. faecium* DH 115; 19, *E. faecium* KH 126; 20, *E. faecium* FH 133 and 21, *L. lactis* subsp. *cremoris* NCDC 61 (negative control). (B) *E. faecalis* 1, *E. faecalis* ATCC 29212 (positive control); 2, *E. faecalis* KH 12; 3, *E. faecalis* KH 62; 4, *E. faecalis* KH 67; 5, *E. faecalis* KH 70; 6, *E. faecalis* KH 72; 7, *E. faecalis* KH 91; 8, *E. faecalis* KH 93; 9, *E. faecalis* KH 98; 10, *E. faecalis* KH 111 and 11, *L. lactis* subsp. *cremoris* NCDC 61 (negative control).

KH 98, FH 99, KH 111, DH 115 and FH 133) showed positive result, which was contradictory to the results of TPM.

3.2. Molecular characterization of *Enterococcus* sp.

The results for the molecular identification of bacteriocinogenic isolates are presented in Figures 2A and 2B. Of the 28 all the 19 biochemically identified *E. faecium* strains were confirmed as *E. faecium* and similarly, all nine *E. faecalis* strains that were identified through biochemical tests were confirmed on molecular basis by the gene-specific PCR.

3.3. Molecular identification of the tyrosine decarboxylase gene (*tdc*)

Primers TD2/TD5 developed by Coton et al. [7] targeting the *tdc* gene allowing for the amplification of a 1100 bp fragment were used in combination with the primers BSF8/BSF1541 (PCR internal control), for the detection of the *tdc* gene. On PCR amplification of all the 28 enterococcal strains, 19 strains (those found positive on TPM) gave two amplicons of 1100 and 1560 bp (for *tdc* gene and internal control, respectively) similar to the positive control, while as expected the negative control only showed amplification of the internal control (Fig. 3). No silent *tdc* gene (i.e. negative in TPM and found to be positive in PCR) was observed in any of the isolates.

3.4. Quantitative analysis of tyramine by HPLC

Quantitative estimation of the tyramine concentration in the fermenting broth under both aerobic and anaerobic incubation was done by the HPLC method. Standard tyramine was used for the standard curve

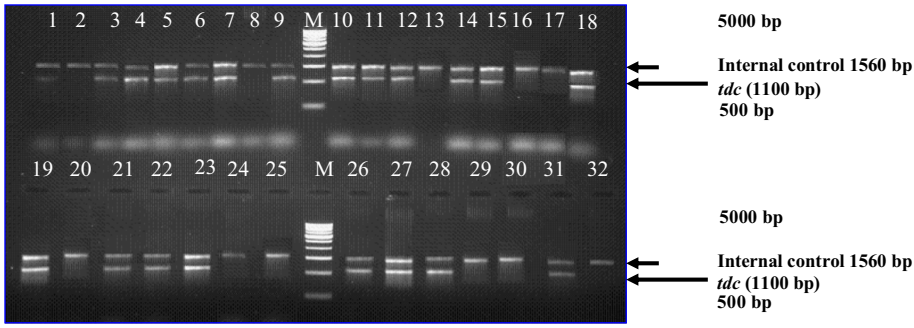

Figure 3. PCR-based detection of tyrosine decarboxylase (*tdc*) gene among different enterococcal strains tested. 1, *E. faecalis* KH 12; 2, *E. faecium* KH 24; 3, *E. faecium* DH 28; 4, *E. faecium* RH 31; 5, *E. faecium* RH 33; 6, *E. faecium* RH 38; 7, *E. faecium* DH 56; 8, *E. faecium* KH 58; 9, *E. faecium* DH 59; 10, *E. faecium* CH 60; 11, *E. faecalis* KH 62; 12, *E. faecalis* KH 67; 13, *E. faecalis* KH 70; 14, *E. faecalis* KH 72; 15, *E. faecium* RH 78; 16, *E. faecium* KH 79; 17, *L. lactis* subsp. *cremoris* NCDC 61 (negative control); 18, *E. faecalis* NCDC 114 (positive control); 19, *E. faecalis* NCDC 114 (positive control); 20, *L. lactis* subsp. *cremoris* NCDC 61 (negative control); 21, *E. faecium* KH 81; 22, *E. faecalis* KH 91; 23, *E. faecalis* KH 93; 24, *E. faecalis* KH 98; 25, *E. faecium* FH 99; 26, *E. faecium* FH 102; 27, *E. faecium* RH 106; 28, *E. faecium* KH 110; 29, *E. faecalis* KH 111; 30, *E. faecium* DH 115; 31, *E. faecium* KH 126 and 32, *E. faecium* FH 133.

preparation. Tyramine standard gave a distinct peak of good resolution in < 3 min of run time. A standard curve was plotted between the peak area and the amount of tyramine. An exact linear curve was obtained with a regression coefficient (r) of 0.991, which indicated a good linear relationship between tyramine concentration and area, hence justifying the acceptability of the gradient elution program used. Nineteen of the 28 enterococcal strains were found to be positive for the production of tyramine. These 19 strains were the same as those found positive on TPM as well as PCR amplification (giving an amplicon of 1100 bp). The levels of tyramine produced by enterococcal isolates ranged from 717 to 9063 $mg{\cdot}mL^{-1}$ and from 512 to 8234 $mg{\cdot}mL^{-1}$ under aerobic and anaerobic conditions, respectively (Tab. I). About 20–40% higher tyramine concentration was observed under aerobic condition as compared to anaerobic condition.

4. DISCUSSION

Tyramine is the most extensively studied BA. This is of bacterial origin in fermented foods and has severe toxicological effects on human health. In the present study, the tyramine production ability was screened among several strains of *Enterococcus* sp. by different methods: decarboxylating media, PCR and HPLC. Of the 28 bacteriocinogenic enterococcal strains, six strains (KH 24, KH 98, FH 99, KH 111, DH 115 and FH 133) showed positive results for tyrosine decarboxylation in the improved medium, i.e. produced a color change, but were found to be negative in TPM, PCR as well as HPLC estimation. Thus, it can be concluded that these strains showed a false positive result on improved medium. Similar, false positive results have also been reported by several workers for this medium [2, 16]. However, three enterococcal strains (DH 28, DH 56 and RH 106) showed

negative results in improved medium i.e. no color change, while, they showed positive results in the case of other three methods. Similarly, a false negative result was also observed by Bover-Cid and Holzapfel [2]. This may be due to the fact that the pH change of the improved medium was too low to change the color of bromocresol purple. Significantly, in the case of TPM no false positives or false negatives were observed because there was no interference with the acidification produced by the fermentation of sugars. As a result, some amount of additional glucose or fructose may be added in TPM for the better growth of microorganisms. All the tested strains grew well in TPM because TPM can provide all the nutrients required for the growth of even fastidious LAB. Moreover, the low glucose concentration, low pH (below 5.5) [21] and the presence of pyridoxal-5-phosphate have a strong enhancing effect on the amino decarboxylase activity [29] and, therefore, an improved tyramine production on TPM plates. Thus, TPM is an easy, conventional and suitable method to screen LAB for tyrosine decarboxylase activity in laboratories lacking sophisticated equipment.

We also evaluated the usefulness of the gene-specific PCR method to detect enterococcal strains possessing *tdc* gene responsible for the production of tyramine. Several *tdc* gene-specific primers are reported for the PCR identification of *tdc* gene [15, 20]. However, we used a set of primers TD2/TD5 [7] for this purpose because they have been shown to give the right response in several species of LAB including *Enterococcus* [5]. Using this set of primers all these 19 test strains and a reference strain that showed positive results on the TPM also gave 1100 and 1560 bp amplification products in the PCR. The presence of amplicon 1560 bp for PCR internal control in all negative strains justifies that the *tdc*-targeted PCR negative results are not due to an inhibition of PCR. We did not observe any silent *tdc* gene (i.e. giving a band of 1100 bp in the PCR, but found to be negative for decarboxylase activity) as observed by Serio et al. [26]. Therefore, PCR results were also in 100% correlation with the results obtained on TPM. Similarly, 100% correlation between the results of TPM and PCR has recently been reported by Landete et al. [16]. Thus, *tdc* gene-specific PCR is an easy and quick genetic tool and provides a rapid means of detecting tyramine-producing microorganism in fermented foodstuffs.

The concentration of tyramine produced by enterococci in the fermenting broth under both aerobic and anaerobic incubation was quantified by the HPLC method. Tyramine production in the broth after three days showed a wide variation, from 717 to 9063 $mg{\cdot}L^{-1}$ (incubated under aerobic conditions), being > 4000 $mg{\cdot}mL^{-1}$ in most of the cases. Relatively a higher tyramine concentration was observed in the case of incubation under aerobic conditions as compared to anaerobic incubation. This is supported by the observations of Landete et al. [16]. Low levels of tyramine under anaerobic incubation may probably be due to the less growth of enterococci as compared to under aerobic incubation. However, levels of tyramine formed by strains of *Enterococcus* sp. in this study were relatively high compared to those previously reported (379–4986 $mg{\cdot}L^{-1}$) in broth [2] and 242–476 $mg{\cdot}mL^{-1}$ in cheese [18]. This variability can be justified by the difference in the composition and the concentration of the precursor in fermenting broth or cheese. Several other factors such as incubation time and temperature may also influence the production of tyramine by these strains.

The present study thus establishes a positive correlation between the results of decarboxylating medium, PCR and HPLC with respect to the production of tyramine by the enterococcal strains. However, the results of the two decarboxylating media are not in agreement with each other. Hence, between the two decarboxylating

media used for the screening of the decarboxylase activity, TPM was found to be better as the results of this medium showed complete correlation with the other two methods. Since the results of HPLC and PCR were found to be showing 100% correlation, these two methods may serve as a complementary approach to attain the same goal. On one hand where HPLC analysis is essential for quantification i.e. determination of the exact concentration of tyramine in the samples, PCR on the other hand is an easy and rapid method for analyzing large numbers of samples for the presence of putative tyramine-producing microorganisms. Moreover, it can also facilitate in the detection of silent *tdc* gene if present in any strain. Thus, all the three methods used in this study are efficient by themselves in one or the other way in the detection of BA.

In the present study as some strains of both *E. faecalis* and *E. faecium* tested were found to be negative for the tyramine production and also, variability was observed in the concentration of tyramine that was produced by the positive strains of both species. Hence, it seems to indicate that the tyramine-producing ability is not related to a particular species of the genus *Enterococcus* or to the genus itself but that it is rather a strain-specific attribute. Recently, Coton and Coton have also shown that the tyrosine decarboxylating ability is not a species-specific trait of *Lactobacillus brevis* but that it is a strain-dependent attribute [6].

Thus, it can be concluded that the tyramine production trait seems to be more common among most strains of genus *Enterococcus* species. As several strains of *Enterococcus* sp. are being used in the food industry as a starter, protective and probiotic cultures, it is, therefore, important that strains used in the food industry must be screened for the production of BAs in order to select the safe strain.

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