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Original Article

Significant production of vanillin and in vitro amplification of *ech* gene in local bacterial isolates

Produção significativa de vanilina e amplificação in vitro do gene *ech* em isolados bacterianos locais

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Abstract

Vanillin is the major component which is responsible for flavor and aroma of vanilla extract and is produced by 3 ways: natural extraction from *vanilla* plant, chemical synthesis and from microbial transformation. Current research was aimed to study bacterial production of vanillin from native natural sources including sewage and soil from industrial areas. The main objective was vanillin bio-production by isolating bacteria from these native sources. Also to adapt methodologies to improve vanillin production by optimized fermentation media and growth conditions. 47 soil and 13 sewage samples were collected from different industrial regions of Lahore, Gujranwala, Faisalabad and Kasur. 67.7% bacterial isolates produced vanillin and 32.3% were non-producers. From these 279 producers, 4 bacterial isolates selected as significant producers were; A3, A4, A7 and A10. These isolates were identified by ribotyping as A3 *Pseudomonas fluorescence* (KF408302), A4 *Enterococcus faecium* (KT356807), A7 *Alcaligenes faecalis* (MW422815) and A10 *Bacillus subtilis* (KT962919). Vanillin producers were further tested for improved production of vanillin and were grown in different fermentation media under optimized growth conditions for enhanced production of vanillin. The fermentation media (FM) were; clove oil based, rice bran waste (residues oil) based, wheat bran based and modified isoeugenol based. In FM5, FM21, FM22, FM23, FM24, FM30, FM31, FM32, FM34, FM35, FM36, and FM37, the selected 4 bacterial strains produced significant amounts of vanillin. A10 *B. subtilis* produced maximum amount of vanillin. This strain produced 17.3 g/L vanillin in FM36. Cost of this fermentation medium 36 was 131.5 rupees/L. This fermentation medium was modified isoeugenol based medium with 1% of isoeugenol and 2.5 g/L soybean meal. *ech* gene was amplified in A3 *P. fluorescence* using *ech* specific primers. As vanillin use as flavor has increased tremendously, the bioproduction of vanillin must be focused.

Key words: vanillin, bacterial production, novel strain, fermentation media, molecular characterization.

Resumo

A vanilina é o principal componente responsável pelo sabor e aroma do extrato de baunilha e é produzida de três formas: extração natural da planta da baunilha, síntese química e transformação microbiana. A pesquisa atual teve como objetivo estudar a produção bacteriana de vanilina a partir de fontes naturais nativas, incluindo esgoto e solo de áreas industriais. O objetivo principal era a bioprodução de vanilina por meio do isolamento de bactérias dessas fontes nativas. Também para adaptar metodologias para melhorar a produção de vanilina por meio de fermentação otimizada e condições de crescimento. Foram coletadas 47 amostras de solo e 13 de esgoto de diferentes regiões industriais de Lahore, Gujranwala, Faisalabad e Kasur; 67,7% dos isolados bacterianos produziram vanilina e 32,3% eram não produtores. Desses 279 produtores, 4 isolados bacterianos selecionados como produtores significativos foram: A3, A4, A7 e A10. Esses isolados foram identificados por ribotipagem como fluorescência A3 Pseudomonas (KF408302), A4 *Enterococcus faecium* (KT356807), A7 *Alcaligenes faecalis* (MW422815) e A10 *Bacillus subtilis* (KT962919). Os produtores de vanilina foram posteriormente testados para produção aprimorada de vanilina e foram cultivados em diferentes meios de fermentação sob condições de crescimento otimizadas para produção aprimorada de vanilina. Os meios de fermentação (FM) foram: à base de óleo de cravo, à base de resíduos de farelo de arroz (resíduos de óleo), à base de farelo de trigo e à base de isoeugenol modificado. Em FM5, FM21, FM22, FM23, FM24, FM30, FM31, FM32, FM34, FM35, FM36 e FM37, as 4 cepas bacterianas selecionadas produziram quantidades significativas de vanilina. A10 *B. subtilis* produziu quantidade máxima de vanilina. Essa cepa produziu 17,3 g / L de vanilina em FM36. O custo desse meio de fermentação 36 foi de 131,5 rúpias / L. Esse meio de fermentação foi um meio à base de isoeugenol modificado com 1% de isoeugenol e 2,5 g / L de farelo de soja. O gene *ech* foi amplificado em A3 *P. fluorescence* usando primers específicos para *ech*. Como o uso da vanilina como sabor aumentou tremendamente, a bioprodução da vanilina deve ser focada.

Palavras-chave: vanilina, produção bacteriana, nova cepa, meio de fermentação, caracterização molecular.

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1. Introduction

Source of natural vanillin is pod of *Vanilla* but it is also present in other plants. In tobacco plant, vanillin is present in traces (Galadima et al., 2019). But the commercial source of natural vanillin is *Vanilla* orchid (Furuya et al., 2015). GPS (Global Product strategy) safety summary revision, the annual consumption of vanillin in 2010 was 12,000 tons. However, less than 1% vanillin is derived from natural source that is from *Vanilla* plant, while remaining is produced by chemical synthesis. The price of vanillin extracted from natural source ranges from 1200$ to 4000$ per KG, while chemically synthesized vanillin cost approximately 15$ per KG (Noort, 2019; Gallage et al., 2014).

Natural production of vanillin is very slow and laborious. It requires hand pollination of flowers. That is why natural vanillin cost is very high (Muheim and Lerch, 1999). Whereas, synthetic vanillin may cause health hazards and is not environment friendly because of many additional chemicals as by products (Banerjee and Chattopadhyay, 2019). These factors increase consumers demand for safe and ecofriendly natural vanillin. In recent years, biotechnological production of vanillin is of great interest (Dubal et al., 2008). According to US and European legislation, biotechnological products can be labeled as "Natural". So, a large number of researches have been carried out in field of biotechnology for bio-production of vanillin (Bicas et al., 2010). Vanillin is a white crystalline solid with a pleasant vanilla like aroma. It is an aromatic aldehyde (3-methoxy-4-hydroxybenzaldehyde) that belongs to phenolic compounds group. General formula of vanillin is $C_8H_8O_3$ and contains many functional groups including aldehyde, ether and phenol.

There is an increased demand for safe, cheaper and "natural" vanillin by consumers which can be fulfilled through biotechnological production of vanillin. The biotechnological process consumes less energy and is safe for consumption. Use of microorganisms for bio-production of vanillin is of great interest. Variety of substrates including eugenol, isoeugenol and ferulic acid are used for vanillin production by bacteria. Various microorganisms are used in bio-production of vanillin from several substrates. Eugenol is converted to vanillin by two step process through various microorganisms such as *Corynebacterium* spp., *Pseudomonas* spp. and *Rhodococcus* spp. By oxidative hydrolysis, eugenol is converted into ferulic acid, an intermediate which is then converted into vanillin through various metabolic pathways (Priefert et al., 2001). Several microorganisms have been reported to produce vanillin from isoeugenol. A bacterial strain *Bacillus fusiformis* SW-B9 produced 32.5 g/L vanillin using 60% isoeugenol which is the highest yield of vanillin from isoeugenol (Zhao et al., 2005; Chattopadhyay et al., 2018; Simon et al., 2014).

Current research work was to develop environment safe protocols to obtain significant production of vanillin. The objectives of the current study were: (i) Screening of vanillin producing bacteria from sewage and soil of local industrial area using specific isoeugenol fermentation medium. (ii) Enhancement of vanillin production from the screened bacterial isolates using various modifications in fermentation media. (iii) Detection of vanillin producing gene enoyl-CoA hydratase/aldolase (*ech)* from selected specific bacterial strains.

2. Materials and Methods

2.1. Sample collection

Various sewage and soil samples were collected from different industries such as chemicals, textiles, ceramics, soap, food and feed. These places have such bacterial strains which have ability to produce vanillin in large quantities because of presence of nutrients required as raw material for vanillin production. During sample collection, autoclaved falcon tubes were used to avoid contamination. Soil and sewage samples were taken from 50mm below the surface. 47 soil and 13 sewage samples collected from different regions of Punjab to isolate bacterial strains. The samples of soil and sewage were taken from a variety of sites (Table 1).

2.2. Sampling from soil

Ten grams of soil samples were separately suspended in 90ml of distilled autoclaved water and then 100µl of each suspension was spread on separate agar plates (nutrient agar plates). The petri plates were kept at 37°C for 24hrs. After incubation, bacterial colonies appeared on plates. These colonies were then streaked on freshly prepared agar medium plates to obtain pure culture.

2.3. Sampling from sewage

Sewage samples were collected in sterilized falcon tubes and 10µl of each of the sample was spread on already prepared nutrient agar plates and incubated at 37°C for 24hrs. Visible colonies appeared after incubation and were then streaked on fresh agar plates.

2.4. Bacterial stock collection

Isolated bacterial glycerol stocks are deposited in microbiology laboratory, department of Zoology, GCU Lahore, Pakistan

2.5. Screening of vanillin producing bacterial strains

Bacterial strains with capacity to produce vanillin were screened using MM9 medium. For primary screening, bacterial isolates were separately grown in 100ml modified medium (MM9). This medium consists of carbon and nitrogen sources which are important for vanillin (glucose 0.5g, $MgSO_4.7H_2O$ 0.5g, $(NH_4)_2SO_4$ 0.2g, $CaCl_2.6H_2O$ 0.032g, KH_2PO_4 0.03g and $Na_2HPO_4.12H_2O$ 0.15g) containing the compounds required for vanillin production (Ashenograph et al., 2011). Isolated bacterial strains were separately grown overnight at 37°C in 100ml MM9 medium containing 1% isoeuginol. pH of the medium used was according to standard parameters. A control experiment was also run with 1% isoeugenol MM9 medium. The biotransformation product (vanillin) was obtained after further 48hrs of incubation. Vanillin was separated by

acidifying (at pH of 2-3 with 10N $H_2SO_{4)}$ and equal quantity of ethyl acetate was added for extraction. The organic layer was separated by centrifugation at 3,000 rpm for 1minute. Then this layer was used for qualitative and quantitative analysis. Initial screening was carried out on 412 bacterial isolates. 279 bacterial strains produced vanillin in reasonable quantities were separated and were proceeded for further studies (Figures 1-4).

2.6. Qualitative analysis: paper chromatography

The vanillin extract was analyzed qualitatively using paper chromatography (Zhao et al., 2005). 0.03M standard solution of vanillin in autoclaved distilled water was prepared. Solvent system of hexane: ethyl acetate (3:4 v/v) was poured in chromatographic glass jar. 50µl of samples and standard vanillin were loaded on chromatographic paper (Whatmann I). Vertically chromatographic

Table 1. Soil and sewage samples collected from variety of sites.

Location	Industry	Soil sample sites	Sewage sample sites
	Chemical	2	1
	Soap	2	-
Industrial area of Kot	Dye	1	1
Lakhpat	Textile	2	1
Lahore Kasur Road	Food	2	-
Industrial area	Feed	2	-
Industrial Estate	Sugar	3	1
Raiwind Road, Lahore	Garments	2	1
Kasur Road Industrial	Chemical	5	1
Estate Lahore	Steel	2	-
Industrial Estate	Chemical	2	1
Faisalabad	Textile	7	2
Industrial Estate-II	Ceramic	3	1
Gujranwala	Plastic	2	1
Garages of Lahore	-	10	2

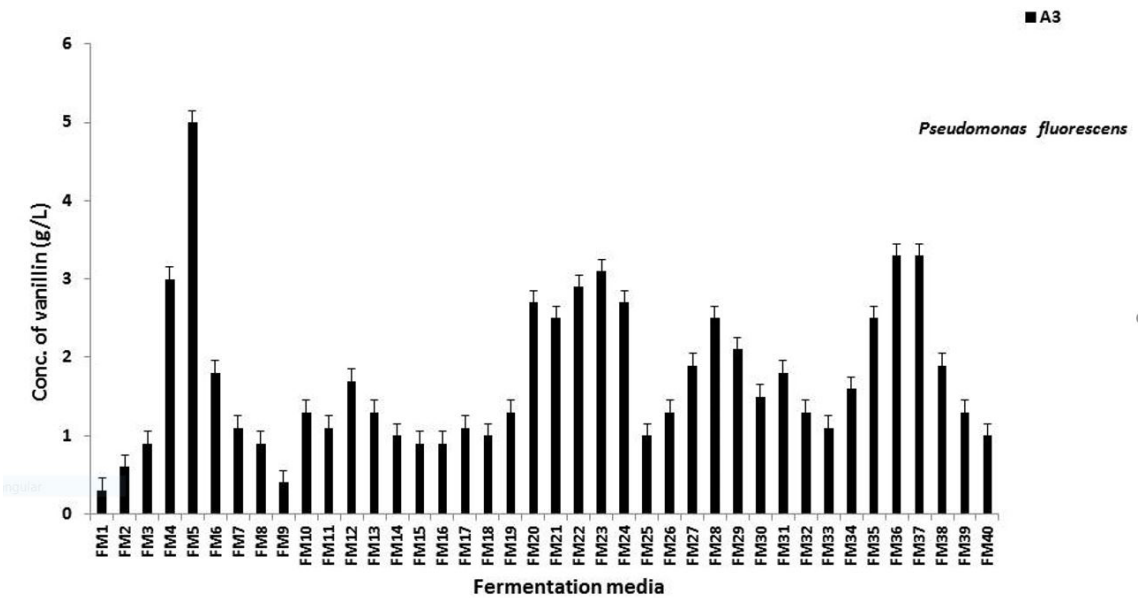

Figure 1. Vanillin production in different fermentation media by strain A3.

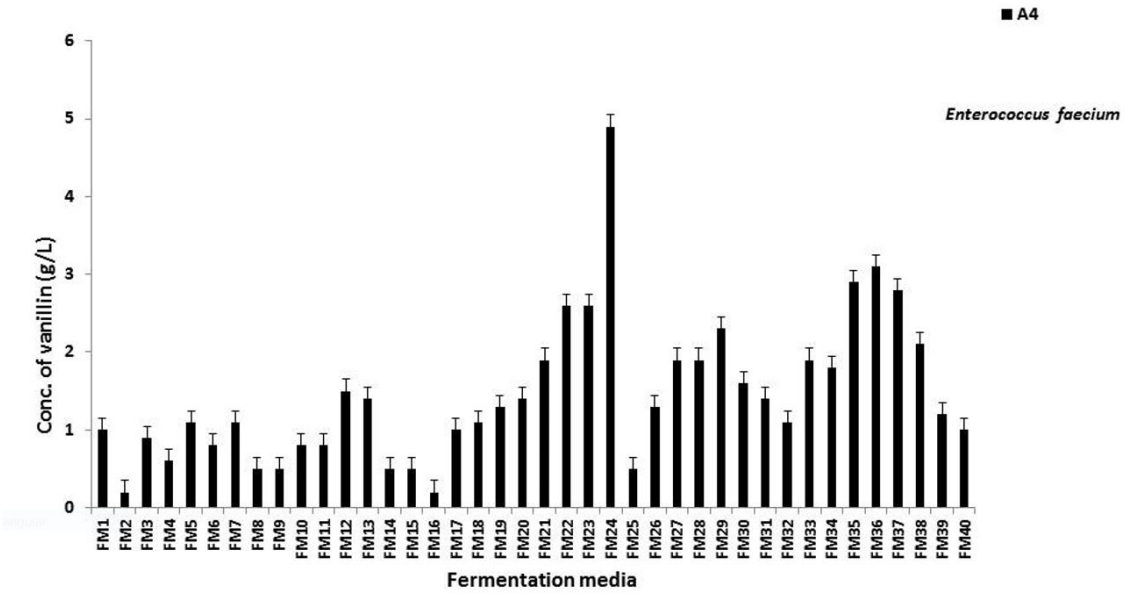

Figure 2. Vanillin production in different fermentation media by strain A4.

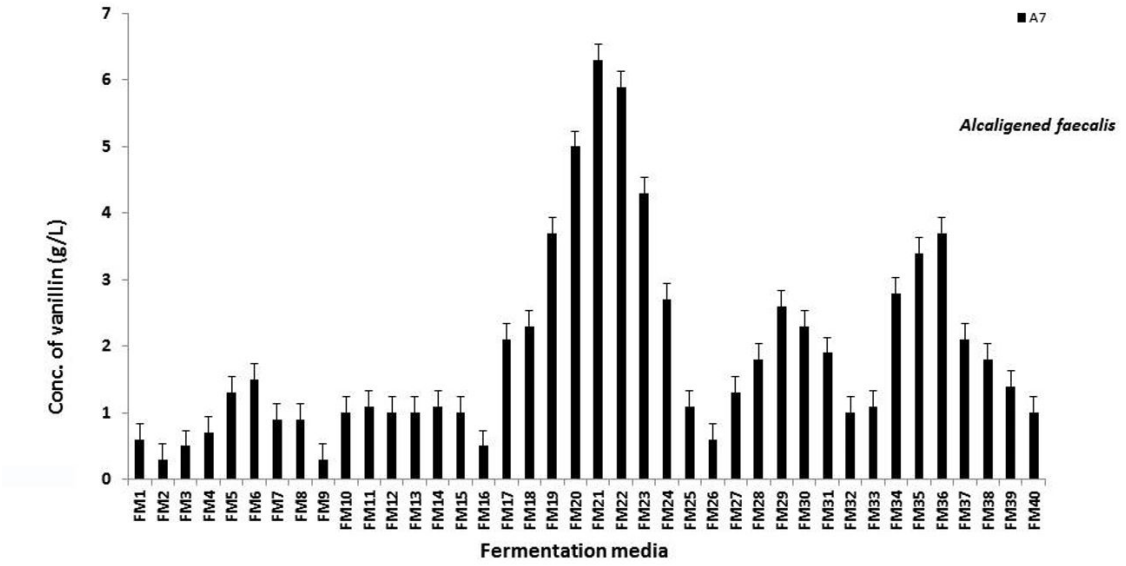

Figure 3. Vanillin production in different fermentation media by strain A7.

papers were irrigated in solvent system (hexane: ethyl acetate) for few hours until solvent reached to a certain point on filter paper. Filter papers were dried at room temperature. Detection of vanillin was done by spraying 0.1% 2-thiobarbituric acid (0.1g/100ml of 2N HCL) and dried at 70 ºC for 10mins to get orange yellow spots. The R_f values of standard vanillin and samples were compared for the confirmation of results. Following Formula 1 was used for calculation of R_f value.

$$R_f = \frac{Distance\ traveled\ by\ vanillin}{Distance\ traveled\ by\ solvent\ system} \quad (1)$$

Distance was measured from point where the vanillin was loaded till the point where solvent was ended (Figure 5).

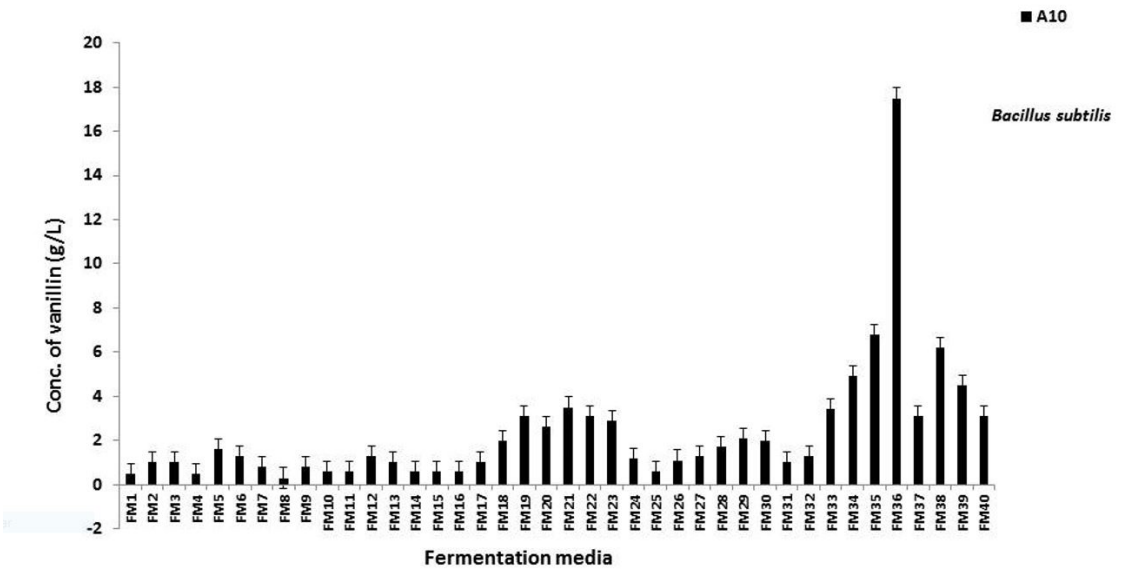

Figure 4. Vanillin production in different fermentation media by strain A10.

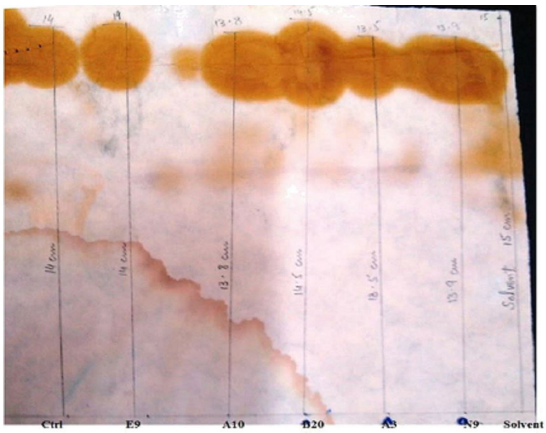

Figure 5. Paper chromatography.

Figure 6. Acidic ninhydrin method.

2.7. Quantitative analysis: acid colorimetric method

Quantitative analysis of vanillin was done as described by Ashengroph et al. (2012). Vanillin was quantitatively determined by the reaction with thiobarbituric acid. Rana et al. (2013) reported that yellow orange color formed when thiobarbituric acid reacts with standard vanillin. Presence of vanillin was confirmed in the culture media sample by formation of orange yellow color (He et al., 1999).

2.8. Spectrophotometric analysis

In 5ml pyrex tubes, 50µl of culture supernatant and 950µl of thiobarbituric acid reagent (500µl 24% HCl, 200µl 1% thiobarbituric acid, 250µl distilled water) were added. Known concentrations of standard solution of vanillin was prepared in same way. Pyrex tubes were kept on water bath for 60 minutes at 55ºC. Then at room temperature tubes were cooled for 20 minutes. Optical density was recorded at 434nm by using PD-303S spectrophotometer. Standard curve was prepared by taking O.D values of known concentration of vanillin at 434nm. Amount of vanillin in liquid broth was calculated from standard curve obtained (Figure 6).

2.9. Physical characterization of vanillin producers

For physical characterization of vanillin producing bacterial strains Gram's staining, motility test and endospore staining tests were performed.

2.10. Biochemical tests

Catalase test, Urease test, Carbohydrate fermentation test, Triple sugar iron test, MRVP test, Citrate test, Oxidase test, Indole test and Blood agar test are the biochemical

tests that were performed to characterize vanillin producers (Benson, 2002).

2.11. Molecular characterization of bacterial strains

Identification of bacterial strains was done by determining 16S rRNA nucleotide sequence (Ribotyping). Genomic DNA was extracted using Phenol-chloroform extraction method. PCR was carried out using universal forward 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primers 1522R (5'-AAGGAGGTGATCCAGCCGCA-3'). Sequencing was done and bacterial isolates were identified (Figure 7 and 8).

2.12. Identification of Vanillin producing gene

A 659 base pair fragment of the gene encoding enoyl CoA hydratase/aldolase (*ech)* was amplified by using *ech* specific primers "(*ech*F: 5'CGGGATCCGGCCGCTGATAGCTACGTTTG-3' and *ech*R: 5'-CGTTCTGCTCCCAGGTCAGCTC-3')". For amplification of *ech* gene, PCR was done. Total volume of reaction mixture was 40 µl. Initial denaturation was done at 94ºC for 2 minutes and short denaturation was carried out at 94ºC for 40 seconds. Then at 65ºC, annealing was done for 45 seconds for *ech* specific primers (optimization). Primer extension was carried out at 72 ºC for 55 seconds and final extension was done at 72 ºC for 10 minutes. By gel electrophoresis amplified PCR products were visualized (Figure 9).

2.13. Sequencing of PCR products

After purification, PCR products of partial sequence of 16S rRNA gene and *ech* gene of bacterial strains were sent to First Base Laboratories, Malaysia for sequencing.

2.14. Detection and quantification of vanillin (analytical methods)

Vanillin produced by screened bacterial strains in isoeugenol based fermentation medium was detected by qualitative analysis using R_f values. The amount of vanillin produced by bacterial strains in screening (isoeugenol based) fermentation medium was analyzed by spectrophotometric method described by Ashengroph et al. (2011).

2.15. Enhanced vanillin production

For enhanced production of vanillin, significant producers (bacterial strains) were grown in various fermentation media. Four bacterial strains were found best producers of vanillin in modified isoeugenol, eugenol and ferulic acid based fermentation media. It has been reported that bacteria usually produce vanillin in µg/L, but in present study, bacteria produced vanillin in mg/L and g/L. The four bacterial isolates were cultured in clove oil based, urea based, wheat bran based, rice bran waste (oil residues) based and modified isoeugenol based fermentation media (Table 2) to obtain vanillin in large amount .

2.16. Statistical analysis

The results were reported as mean ± standard deviation (SD) and analyzed by one way ANOVA. p value was less

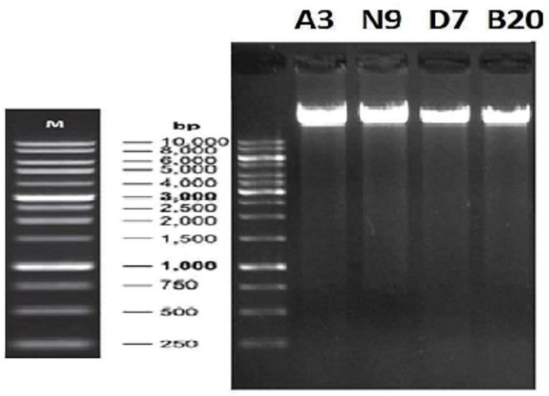

Figure 7. Genomic DNA isolation.

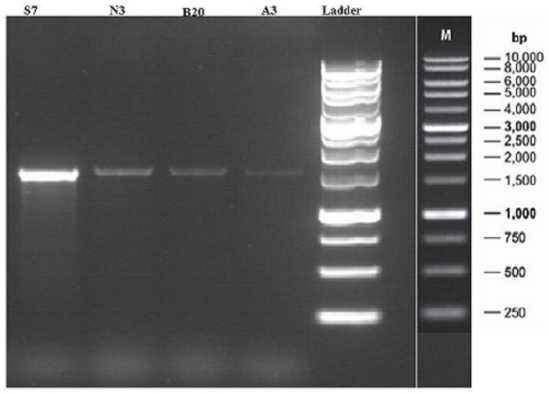

Figure 8. PCR product of 1500bp.

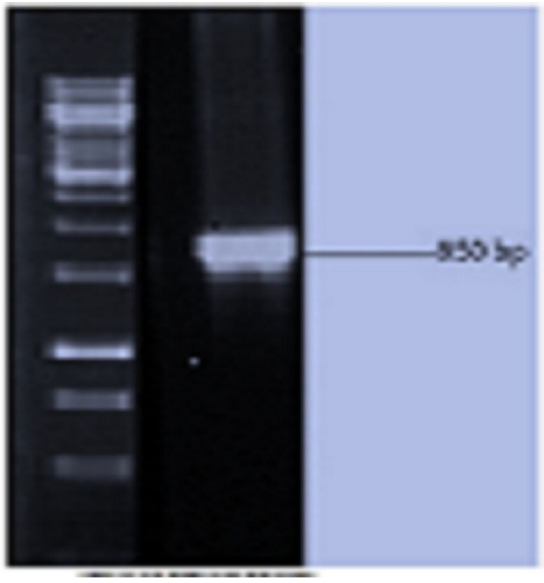

Figure 9. PCR product of *ech* gene (550 bp).

than 0.05 which showed significant increase in production of vanillin by bacteria.

3. Results

A total of 412 bacterial strains were isolated from 47 soil and 13 sewage samples collected from different industrial regions of Punjab. After primary screening, 133 bacterial isolates were non-producers while 279 strains showed the ability to produce vanillin. Four bacterial strains *i.e.*, A3 *P. fluorescence*, A4 *E. faecium*, A7 *A. faecalis* and A10 *B. subtilis* showed production of vanillin in isoeugenol based fermentation medium which was above 1g /L (Table 3).

3.1. Production of vanillin in clove oil based fermentation medium by bacterial strain A3 P. fluorescence (KF 408302)

Strain A3 *P. fluorescence* was isolated from soil sample of feed industry of Lahore and Kasur road industrial areas. Different morphological and biochemical tests were performed to characterize A3 strain. After ribotyping, A3 strain was identified as "*P. fluorescence*". *P. fluorescens* contains multiple flagella and it can grow in soil and water. It has versatile metabolism. A3 showed positive citrate, indole, catalase and starch hydrolysis tests. .

This strain has ability to produce vanillin in different fermentation media. 0.2 g/L is the lowest amount of vanillin produced by A3 in fermentation medium, while showed maximum production of vanillin which is 5.0 g/L in FM5 in which 1% clove oil was added. Significant production of vanillin was observed after 96 hours of incubation and harvest pH was 7.0. Cost of FM5 was Rs. 13.5/- per litre (Table 3).

3.2. Production of vanillin in wheat bran based fermentation medium by bacterial strain A4 E. faecium (KT356807)

Bacterial strain A4 *E. faecium* was taken from sewage sample from a garage in Mazang, Lahore. Various morphological and biochemical test were performed to characterize A4 strain. It is a Gram positive, non-hemolytic bacterium which is used as probiotics in animals and humans. After ribotyping, A4 was identified as "*E. faecium*". *E. faecium* (KT356807) showed negative tests for indole and catalase (Table 4). This bacterial strain can ferment glucose to acid and is also able to ferment lactose.

Strain A4 showed different amount of vanillin production in different fermentation media. It showed maximum production of vanillin in FM24 which is 4.9 g/L in which 500 g/L wheat bran was added and 0.1 g/L vanillin production in FM1 which is lowest production of vanillin by this strain. Strain A4 showed significant production of vanillin after 96 hours of incubation and 6.5 was harvested pH. Cost of FM24 was 131.9 rupees per litre.

3.4. Production of vanillin in wheat bran based fermentation medium by bacterial strain A7 A. faecalis (MW422815)

Bacterial strain A7 *A. faecalis* was collected from sewage sample of dye industry and Industrial area of

Table 2. Amount of ferulic acid fraction produced from rice bran waste (oil residues).

	Rice bran							
waste (oil residues)(g)	15	20	30	35	40	50	55	60
Ferulic acid fraction produced (g)	1.33	1.9	2.38	2.54	2.78	3.26	3.68	4.0

Table 3. Significant production of vanillin by different strains in different fermentation media.

Strain	Vanillin produced (g/L)	pH	Incubation period
A3	FM5 (5.0)	7.0	96hrs
A4	FM24 (4.9)	6.5	96hrs
A7	FM21 (6.1)	7.0	96hrs
A10	FM36 (17.3)	7.0	96hrs

Table 4. Morphological and biochemical characterization of bacterial isolates.

Morphological/ biochemical	Bacterial strain			
tests	A3	A4	A7	A10
Shape	Rod	Cocci	Rod	Rod
Gram's staining	-tive	+tive	-tive	+tive
Motility	Motile	Non-motile	Motile	Motile
Endospore staining	No spore	No spore	No spore	+tive
Blood agar	+tive	-tive	+tive	+tive
Methyl red	-tive	-tive	-tive	+tive
Indole	-tive	-tive	-tive	-tive
VP	-tive	+tive	-tive	+tive
Oxidase	+tive	-tive	+tive	-tive
Catalase	+tive	-tive	+tive	+tive
Citrate	+tive	+tive	+tive	-tive
Urease test	-tive	-tive	+tive	-tive
Glucose fermentation	-tive	+tive	+tive	-tive
Sucrose fermentation	-tive	+tive	+tive	-tive
Lactose fermentation	-tive	+tive	+tive	-tive

Kot Lakhpat. It is a motile, Gram negative rod shaped bacteria. Strain A7 was characterized by morphological and biochemical tests and after ribotyping, it was identified as *A. faecalis*(Figures 7-9). *A. faecalis* (A7) showed positive results for nitrate reduction, oxidase, catalase, citrate and alpha hemolytic tests (Table 4). In general this bacterium is considered as non-pathogenic. It is obligate aerobe and has ability to degrade urea and increase pH of environment. *A. faecalis* shows alkali tolerance and maintain neutral pH in its cystol.

Strain A7 showed ability to produce vanillin in different fermentation media. Its minimum production of vanillin was in FM2 which is 0.2 g/L while it showed maximum production of vanillin in FM21 which is 6.1 g/L. In FM21 300 g/L wheat bran was used to enhance vanillin production. This strain showed significant production of vanillin after 96 hours of incubation with harvested pH 7. The FM21 in which A7 showed maximum production of vanillin costs 92.9 rupees per litre.

3.5. Production of vanillin in modified isoeugenol based fermentation medium by bacterial strain A10 B. subtilis (KT962919)

Strain A10 *B. subtilis* was collected from auto mobile garage in Iqbal Town Lahore. It was characterized by morphological and biochemical tests. After ribotyping it was identified as "*B. subtilis*". *B. subtilus* is a rod shaped, Gram positive bacteria, which is more common in soil and gastrointestinal tract of animals. It is facultative aerobe and endospore forming bacteria. *B. subtilus* is widely used in biotechnological industry due to high production of enzymes. Strain A10 *B. subtilus* (KT962919) is catalase positive and urease negative (Table 4). Strain A10 *B. subtilus* (KT962919) showed ability to produce vanillin in different fermentation media. It showed maximum production of vanillin in FM36 which is 17.3 g/L while its minimum production of vanillin was 0.3 g/L vanillin in FM8. In FM36 1% isoeugenol (10ml/L) was added. Significant production of vanillin was observed after 96 hours of incubation at pH 7. Cost of FM36 was 131.5 rupees per litre. In this fermentation medium, bacterial strain A10 showed maximum production of vanillin.

3.6. Identification of vanillin producing gene

In bacterial strain A3 *P. fluorescence* a 659-bp fragment of the gene encoding enoyl-CoA hydratase/aldolase (*ech)* was amplified by using *ech* specific primers . This gene is involved in biosynthesis pathway of vanillin in *P. fluorescence*.

4. Discussion

Less than 0.25% vanillin originates from natural sources, more than 97% produced chemically (80-85%) by guaiacol, 12-14% from lignin containing waste and less than 1% is synthesized from microorganisms (Di Gioia et al., 2011; Fleige et al., 2013). World's demand for natural vanillin cannot be met by vanilla extracts from *Vanilla planifolia* and *Vanilla tahitiensis* plants because of high price of vanilla beans and limited production of natural vanilla (Bicas et al., 2010). So there is an increased consumer's demand for natural vanillin (Li et al., 2008). Some bacterial strains have ability to produce vanillin in large quantities (Ashenograph et al., 2011; Berger, 2007).

Moreover, the production of vanillin is enhanced by use of suitable fermentation media and genetic engineering. Not only food and cosmetic industries but also many environmental groups have favored production of vanillin from biotechnological routes. Biotechnological production of vanillin is an alternative to natural vanillin production from *Vanilla* plant.

In the last two decades major research activities have been carried out on bioconversion of natural products like ferulic acid, isoeugenol and eugenol to vanillin using microorganisms at industrial level. Aim of the research work was to check vanillin production from locally isolated bacterial strains. Natural products like isoeugenol, clove oil, wheat bran and waste residues of rice bran oil were used as sole carbon source by wild bacterial strain for significant production of vanillin. Also that increased knowledge of identification and characterization of vanillin producing genes and metabolic pathways involve in vanillin production can be helpful in bioengineering of industrially applicable microorganisms for vanillin synthesis. Many microbial species have been reported to convert ferulic acid to vanillin like *Pseudomonas* spp., *Bacillus* spp., *Staphylococcus* spp. and *Enterococcus* spp. (Mathew and Abraham, 2006; Banerjee and Chattopadhyay, 2019). As currently Pakistan is importing vanillin from China and is not producing at its own which actually costs quite an amount resulting in burden at economy of Pakistan. Third world countries battling with poverty and economy crisis like Pakistan must not spend money on import. Rather it should be able to economically produce the vanillin and export it to other part of world. It may help in its growing economy. Chemically synthesized vanillin has issues regarding safety and health conditions of consumers. There is need to explore local resources to isolate native bacteria who are good producers of vanillin and can be used at industrial level. This will not only help in consumer health friendly vanillin but also will lessen the economic burden. There is ultimate need of improved techniques and methodologies for exploration and characterization of vanillin production encoding genes (Pugh et al., 2015). Specific metabolic pathways involved in vanillin production in these native bacterial isolates can be used as helpful tool in bioengineering of new microorganisms for vanillin synthesis in food and other industries.

5. Conclusion

From this study, it can be concluded that using different fermentation media and optimized growth conditions, these native strains can be used for production and further studies in order to improve production at commercial level in economical and cheaper ways.

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