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Optimization of Fermentation Conditions for Bioactive Compounds Production by Marine Bacterium *Enterococcus Faecium*

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Abstract: Thirty four bacterial strains were isolated from sea water, sediments and algae samples which were collected from Alexandria beaches. All strains were screened for their potentiality to produce bioactive compounds by using well cut diffusion technique against the following pathogens: *Staphylococcus aureus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus* and *Candida albicans* as indicator strains. The most potent strain was identified at the molecular level as *Enterococcus faecium*, while the most susceptable strain was *S.aureus*. Well cut diffusion technique was performed using different culture media (nutrient agar, Zobell agar and Luria Bertani), the most suitable medium was Luria Bertani with inhibition zone of 10 mm. Placket-Burman design was applied to optimize the fermentation conditions and maximize the productivity. The optimized medium was formulated as follows: (g/l): peptone, 15; yeast extract, 2.5; concentrated sea water (>100%), adjusted to pH 8 and inoculum size 1.5 ml, this medium gives inhibition zone of 16 mm when incubated at 35°C for 48 h i.e inhibition zone was increased about 1.6 fold increase. Mutation techniques (physical and chemical) were applied to increase bioactive compound productivity but reverse effect was detected. Immobilization using both entrapment (alginate) and adsorption (luffa and pumice) techniques were applied. Only cells adsorped on pumice gave higher productivity and the inhibtion zone reached up to 17 mm.

Key words: Bioactive compounds – bacteria- inhibition zone- mutation –immobilization .

INTRODUCTION

Bacteria and other micro-organisms are ubiquitous in the marine environment. They are taxonomically diverse, biologically active, and colonize all marine habitats, from the deep oceans to the shallowest estuaries [44]. It has been estimated that the majority of bacteria in natural aquatic ecosystems are organized in biofilms [12]. In a biofilm, a microbial community is attached to a surface and embedded in a self-produced matrix composed of extracellular polymeric substances. This structure provides the bacteria present in the biofilm with several advantages compared to those living as planktonic cells. First, the bacteria are maintained in the selected micro environment where population survival does not depend on rapid multiplication [30]. This is especially advantageous in environments where the bacteria are exposed to constant liquid movements, as, for example, in aquatic environments. Additionally, the bacterial cells present in a biofilm have an increased resistance to desiccation, grazing, and antimicrobial agents compared to their planktonic counterparts [22,38]. Also, biofilms offer enhanced opportunities for interactions such as horizontal gene transfer and co-metabolism [30,48].

The occurrence of large scale of bioactive compounds is not common to all living organisms, but restricted to certain taxonomic groups. Recent research progresses reported that many bioactive natural products from marine invertebrates have striking similarities to metabolites of their associated micro-organisms including bacteria [43]. Compared with terrestrial organisms, the secondary metabolites produced by marine organisms have more novel and unique structures owing to the complex living circumstance and diversity of species, and the bioactivities are much stronger [46].

Competition among microbes for space and nutrient in marine environment is a powerful selection pressure that endows marine micro-organisms to produce marine natural products possessing medical and industrial values [3]. Many antimicrobial, antifouling substances

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have been found among these kinds of bacteria due to the specialized role they play in their respective hosts [26]. It is suggested that the primary role of these antibiotic substances could be related to ecological competition [8].

Different applications of bacteria as probiotics have been evaluated in vitro and in vivo for their potential to inhibit fish and larvae in aquaculture [19] for example *Carnobacterium* [40] and *Enterococcus* spp. [50,39]. Other applications of bacteria including the production of antifouling agents have been studied [11].

The nutritional and environmental conditions have a great influence on production of the antimicrobial substances [34]. In order to develop an efficient production of antimicrobial substances, knowledge regarding the environmental factors affecting this process needs to be well identified. Experimental designs are excellent techniques for optimization of culture conditions to achieve optimal production [14,15].

The aim of this work was isolation of some marine bacteria capable of producing bioactive compounds and optimizing the fermentation conditions for maximum production.

MATERIALS AND METHODS

Organisms and Maintainance: Thirty four bacterial strains were isolated from sea water, sediments and algae samples which were collected from Alexandria beaches on nutrient agar medium. All strains were screened for their potentiality to produce bioactive compounds against the pathogen *Staphylococcus aureus* (ATCC 6538), *Streptococcus faecalis* (ATCC 8043), *Pseudomonas aeruginosa* (ATCC 8739), *Escherichia coli* (ATCC 8739), *Micrococcus luteus* (ATCC 10240) and *Candida albicans* were used as indicator strains. These indicators were kindly provided by Dr.Wefky, S.H. (National Institute of Oceanography, and Fisheries, Alex.Egypt). The most promising strain was chosen and identified according to the standard procedures described by [47,49,52]. Moreover it was subjected to molecular identification.

Antagonistic Action Against Indicator Microorganisms: The well-cut diffusion technique was used to test the ability of the bacterial isolates to inhibit the growth of indicator bacteria and yeast, 50µl was add in each well, After incubation period, the radius of clear zone around each well (Y) and the radius of the well (X) were linearly measured in mm, where dividing Y^2 over X^2 determines an absolute unit (AU) for the clear zone. The absolute unit of each antagonistic isolate, which indicates a positive result in the antagonistic action, was calculated according to the following equation [16]: $AU= Y^2/X^2$

All experiments were done in triplicates and the average was calculated.

Molecular Characterization:

Isolation of Bacterial DNA: The genomic DNA of the most promising producer S14 was isolated from overnight cultures according to the method described by Sambrook *et al*. 1989. Cells were collected by centrifugation and re-suspended in 500 µl TEN buffer. After incubation at 37°C for 30 min, 30 µl of 10% Sodium Dodecyl Sulphate SDS were added and the tubes were inverted gently several times till complete lyses. An aliquot of 5 mg/ml of proteinase K added and the tubes were incubated at 37°C for one hour. After incubation the solution was phenol-extracted several times to remove the protein and once with chloroform to remove the phenol traces. The DNA was precipitated using 0.8 volume of isopropanol and washed with 70% ethanol. The DNA was dried and dissolved in 10 mM Tris HCl, pH 8 and stored at 20°C.

Amplification of 16S rRNA Gene: The 16S rDNA was amplified by polymerase chain reaction (PCR) using the primers 16F27 and 16R1492. Approximately a 1500-base pair fragment of 16S rDNA region was amplified according to the *Escherichia coli* genomic DNA sequence. Amplicons were obtained with a PCR cycling program of 94°C for 1 min followed by 30 cycles of denaturating at 94°C for 1 min, annealing at 55°C for 1 min and polymerization at 72 for 2 min. At the end of thermocycling, The PCR reaction mixture was incubated at 72°C for 10 minutes. As described by [4]. amplicons were visualized by electrophoretic separation on 1% agarose gels stained with ethidium bromide. PCR fragments were purified from amplification reactions with QIA quick PCR purification reagents (QIAEN) according to the kit manual.

Sequencing of PCR-DNA Product: DNA sequence was obtained using DNA sequencer (ABI 310). The PCR product was sequenced using the same PCR primers and other internal primers to confirm the sequence.

Sequence Similarities and Phylogenetic Analysis: Blast program (www.ncbi.nlm.nih.gov/blast) was used to asses the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software [23]. The phylogenetic tree was displayed using the TREEVIEW program [41].

Effect of Culture Medium Type on Production of the Bioactive Compounds: The selected bacterial

strain was grown using nutrient agar, ZoBell and Luria Bertani (LB) media (prepared with sea water) at 30°C for 24 hrs. Culture cell free supernatant was tested against the previously chosen indicator organisms using agar well diffusion technique aiming to obtain the highest productivity [1].

Evaluation of Nutritional Factors: The Plackett-Burman design [42,53]. was applied to reflect the relative importance of various factors involved in the production of these bioactive compounds by the chosen strain. For each variable a high (+) and low (-) levels were tested. The examined variables in this experiment and their levels are shown in Table 2. Eight different trials were performed in duplicates. Rows in Table 3 represent the different trials (row no. 9 represents the basal control). The main effect of each variable was determined with the following equation:

$$Exi = (Mi+ - Mi-) / N$$

Where Exi is the variable main effect, and Mi+, Mi- are the radius of the clear zone around each well in the trials, where the independent variable was present in high and low concentrations, respectively, and N is the number of trials divided by 2. Statistical t-values for equal unpaired samples were calculated using Microsoft Excel to determine the variable significance.

Effect of Mutations on Bioactive Compounds Production: To study the effect of mutations on the productivity of the selected strain against the different pathogens, physical mutation was performed using UV light (254 nm) at different time intervals in order to plot the survival curve. Induction of mutation was carried out at a dose that yielded 90 % mortality as determined from the previous survival curve following the procedure of Kung & Lee [33]. To study the effect of chemical mutation, ethidium bromide was used as chemical mutagen according to [21]. Each mutant was tested for its potent ability against the previously mentioned pathogens using the well cut diffusion technique.

Effect of Immobilization on Bioactive Compounds Production: Immobilization was performed using both entrapment and adsorption techniques as was described by Eikmeier & Rehm, [13] aiming to enhancing the production of the bioactive compounds against different pathogens.

Electron Microscopy: The adhesion of the selected bacteria on or in the supporting materials and formation of the biofilm was carried out using scanning electron microscope. Cells grown in LB were harvested by centrifugation, washed with phosphate buffer and fixed with 2 % glutaraldehyde followed by 1 % osmium tetroxide treatment. After complete fixation, samples were washed in buffer solution, and then dehydrated in ascending order of ethanol concentrations. The samples were dried completely in a critical point dryer, and finally coated with gold in JEOL-JFG1100 E ion-sputter-coater. The specimens were viewed in JEOL-JSM 5300 microscope operated at 20 kV with a beam specimen angle of 45°.

RESULTS AND DISCUSSION

Results: The marine isolate S14 was the most promising strain, it inhibited the growth of all pathogens especially *Staphyllococcus aureus*, the inhibition zone was (9mm) on sea water agar medium.This strain was isolated from the surface of the alga (*Ulva sp.*) from Sidi-Bishr. It is biochemically identified as *Enterococcus sp.* This was confirmed using the molecular techniques .

Molecular Characterization of *Enterococcus* Species: DNA of the promising *Enterococcus* sp. was extracted and the extracted 16S rRNA gene was amplified using the universal primers 16F27 and 16R1492. The produced amplicons was analyzed using agarose gel electrophoresis as shown in Figure 1. It was clear that this strain showed nucleotide size of 1490 base pair compared with phage λ DNA *Hind* III cut molecular weight marker.

Sequencing of PCR-DNA Products: The amplified DNA was sequenced using ABI 310. The sequencing data obtained utilizing this strategy was 500 base pair which represents the partial coding sequence of 16S rRNA gene. The sequencing data was analyzed using nucleotide BLAST search computer based program where this sequence was compared with that of any other rRNA (or rDNA) genes that have been sequenced so far. The resulting data indicated that the isolate under study was identified as *Enterococcus faecium* with identity percentage 92% which confirms the identification using the traditional biochemical tests. The phylogenetic tree was displayed using the TREEVIEW program as shown in Figure 2

Optimization of Bioactive Compounds Production by *E. Faecium*:

Effect of Medium Type on the Production of the Bioactive Compounds: *E. faecium* was examined for the antagonistic activity using three different media; nutrient agar (NA), Luria Bertani (LB) and ZoBell sea water agar . As shown in Table 1, the highest activity was observed using LB.

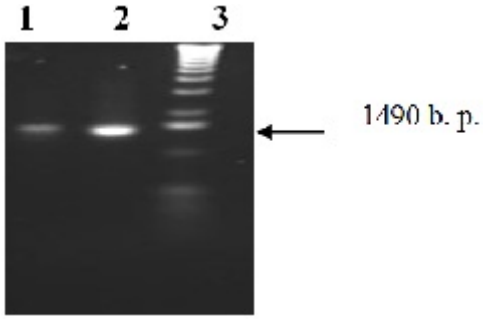

Fig. 1: 16S agarose gel electrophoreses of the extracted and amplified DNA. Lanes1& 2= purified PCR products of the isolate, and lane 3 is phage λ DNA Hind III cut molecular weight marker.

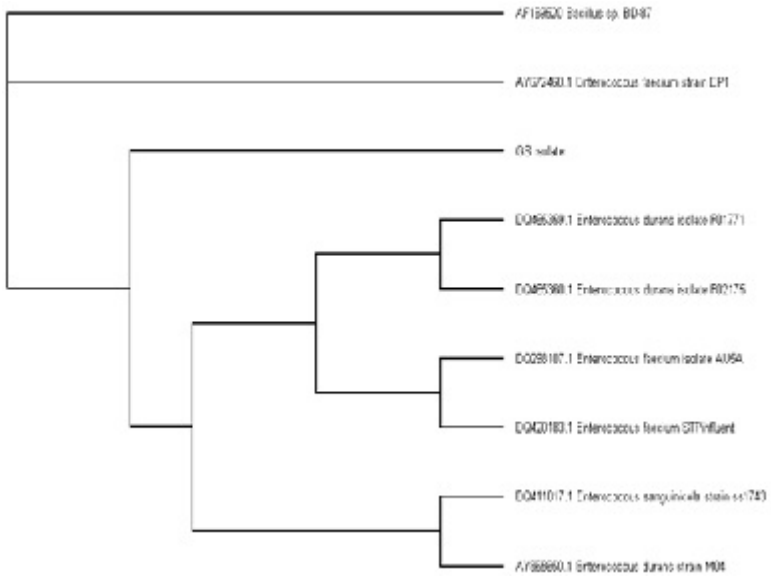

Fig. 2: Phylogenetic relationships among representative experimental strain and the most closely related *Enterococcus* speciesThe dendogram was generated using Tree View Program.

Table 1: Effect of the growth media on the production of the bioactive compounds by *E. faecium*.

Inhibition zone (mm) produced by the selected strain using different media		
LB	NA	ZoBell
10	9	9

Optimization of the Fermentation Factors: The Plackett-Burman design was applied to reflect the relative importance of various factors involved in the production of these agents by *E. faecium* Ep1 .The main effect of each variable on the production of the bioactive compounds as well as *t-values* were estimated for each independent variable as shown in Table 4 and graphically presented in Figure 3. Results in this Figure indicated that the main effect of all variables were positive on the production by *E. faecium* except for yeast extract where high concentration of yeast extract in the medium causes decrease in the production. Statistical analyses of the results (t-test) showed that variations in yeast extract and the incubation period in the tested ranges had the most considerable effects on the production of bioactive compounds by *E. faecium*.

The interacting effect of yeast extract with the incubation period is described in three-dimentional representation (Figure 4). As illusterated, the inhibitory effect of high levels of yeast extract on the antagonistic activity of *E. faecium* can be partially overcome by preparing cultures with decreasing level of yeast extract and extending the incubation period.

According to the obtained results, the predicted medium for cultivation of *E. faecium* to enhance maximum production of the bioactive compounds was formulated as follows: (g/l): peptone, 15; yeast extract, 2.5; concentrated sea water (>100%) , adjusted to pH 8 and inoculum size (1.5 ml for each 50ml medium) all of which are incubated for 48 h at 35°C.

In order to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was applied to compare between the predicted near optimum levels of independent variables

and the basal condition settings. Results in Table 5, confirmed that,the production of the bioactive compounds increased and the inhibition zone increased by 1.6 fold increase.

Table 2: Independent variables affecting production of the bioactive compounds and their levels in the Plackett- Burman design

Factor	Symbol	Level		
		-1	0	1
Peptone (g/l)	P	5	10	15
Yeast extract (g/l)	Y	2.5	5	7.5
Inoculum's size (ml)	IS	0.5	1	1.5
Sea water concentration (%)	C	50%	100%	>100%*
pH	pH	6	7	8
Temperature (°C)	T	25	30	35
Incubation period (h)	IP	12	24	48

*150 ml of sea water was concentrated to100 ml by evaporation

Table 3: Experimental results of the Plackett- Burman design

Trials	Factors symbols							(Response) Diameter of inhibition zone
	P	Y	IS	C	pH	T	IP	(mm)
1	-1	-1	-1	1	1	1	-1	11
2	1	-1	-1	-1	-1	1	1	16
3	-1	1	-1	-1	1	-1	1	0
4	1	1	-1	1	-1	-1	-1	0
5	-1	-1	1	1	-1	-1	1	14
6	1	-1	1	-1	1	-1	-1	12
7	-1	1	1	-1	-1	1	-1	0
8	1	1	1	1	1	1	1	15
9	0	0	0	0	0	0	0	10

Table 4: Statistical analysis of the Plackett-Burman experimental design

Variable	Main Effect	*t-value*
Peptone	4.75	0.8
Yeast extract	-9.75	-2.4
Inoculum size	1.75	0.36
Sea water conc.(%)	3.25	0.6
pH	2.75	0.55
Temperature	4.25	0.76
Incubation period	5.75	1.09

t-value significant at the 1% level = 3.70
t-value significant at the 5% level = 2.45
t-value significant at the 10% level = 1.94
t-value significant at the 20% level = 1.37
Standard *t-values* are obtained from Statistical Methods (Cochran and Snedecor, 1989).

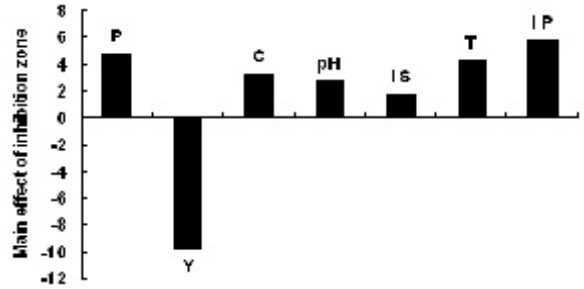

Fig. 3: Elucidation of fermentation conditions affecting the production of the antagonistic agents of *E.faecium*.

Effect of Mutation on Bioactive Compounds Production: *E. faecium* was subjected to two types of mutations (physical and chemical) to investigate the productivity of bioactive compounds. It was subjected to irradiation with UV lamp at 254 nm as a physical mutagen and ethidium bromide as a chemical mutagen.Variants obtained from both types of mutation were tested for the production of bioactive compounds to select the most potent one.

Concerning the physical mutation by UV irradiation, a survival curve was plotted using survival %of the treated strains against the exposure time (h) as shown in Figure 5 . Exposure time at which about 90% lethality of the bacterial population was 1 min. Capacity of bioactive compounds production was determined for each mutant.Comparison between the wild type and its variants is presented in Table 6. Results indicated that UV-mutant of *E. faecium* exhibited lower activity (13 mm inhibition zone) compared to its wild type (16 mm) while ethidium bromide-mutant was better than UV-mutant (15 mm) but still lower than the activity of the wild type.

Effect of Immobilization on Bioactive Compounds Production by *E. Faecium*: Living cells of *E. faecium* Ep1 were subjected to immobilization using adsorption and entrapment techniques. Adsorption was carried out using both luffa pulp and pumice as supporting materials. Figure 6 showed the adsorption of the cells on luffa pulp and pumice while entrapment was done using sodium alginate as a gel matrix. The aim of this experiment is to compare the production of antimicrobial agents by both the free and immobilized cells of *E. Faecium*. The optimized medium containing the adsorbed cells of *E. faecium* was used. Results in Table 7 revealed that *E. faecium* biofilm on pumice showed relatively higher activity in the production of the bioactive compounds (1.2 fold increase) in the diameter of the inhibition zone.

Using luffa pulp as the supporting material decreased the production to about 0.81 fold in the inhibition zone and about 0.66 fold in the activity unit compared to the free non-fixed cells. By using entrapped cells of the wild *E. faecium*. Results indicated a decrease in the inhibition zone diameter to about 0.75 fold and about 0.56 in the activity unit compared to the free cells.

Discussion: Marine organisms are a rich source of structurally novel and biologically active metabolites [7,18,]. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry [20]. To date, many chemically unique compounds of marine origin with various biological activities have been isolated,

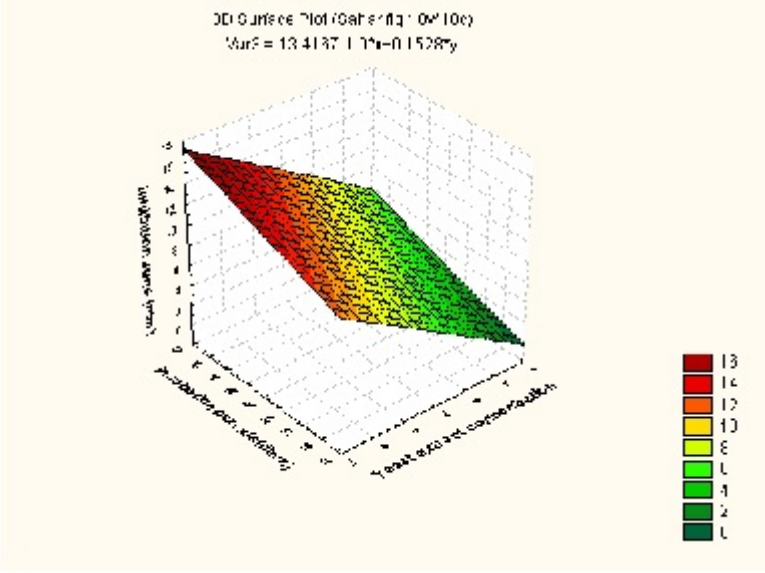

Fig. 4: The interaction effect of yeast extract concentrations (g/l) with incubation periods (days) Levels , with respect to inhibition zone (mm) based on Plackett-Burman results.

Table 5: A verification experiment showing antagonistic activity of *E. faecium* grown on basal against optimized media

Inhibition zone (mm)	Basal medium	optimized medium
	10	16
Activity unit (AU/ml)	4	10.24

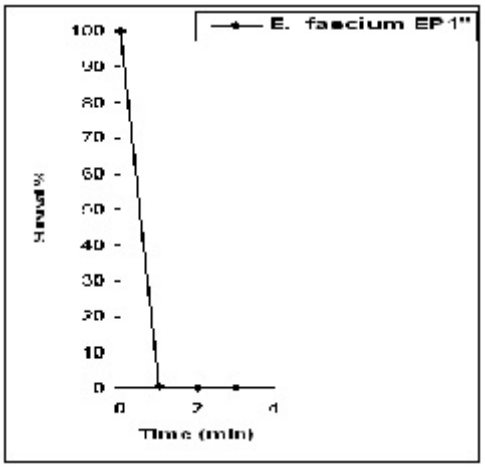

Fig. 5: Survival curves of *E. faecium* treated with UV irradiation.

Table 6: Comparison among the wild type and their mutants for production of the bioactive compounds

E.faecium	Wild type	Mutant (UV)	Mutant (ET)
Inhibition zone (mm)	16.0	13.0	15.0
Activity unit (AU/ml)	10.24	6.76	9.0

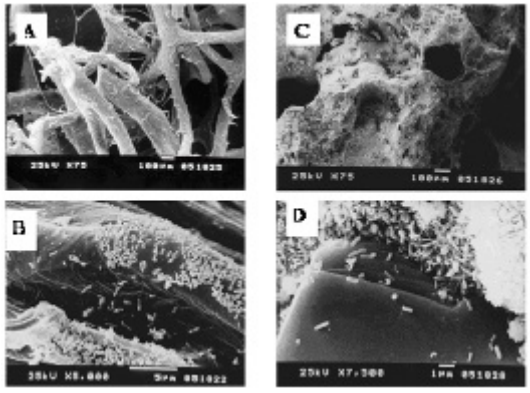

Fig. 6: Scanning electron micrographs showing (A) control luffa pulp; (B) the wild *E. faecium* adsorbed on luffa pulp; (C) control pumice and (D) the wild *E. faecium*

and some of them are under investigation and are being used to develop new pharmaceuticals [20,35]. The ability of marine bacteria to produce secondary metabolites of potential interest has been extensively documented.

The most promising isolate S14 (*Enterococcus* sp.) identified using molecular techniques. Recently, 16S rRNA sequence comparison has been used as a powerful tool for establishing phylogenetic and evolutionary relationships among organisms [37]. An approach presently employed in many laboratories uses

Table 7: Effect of immobilization on production of the bioactive compounds by the *E. faecium* cells

Bacterial strains	Antagonistic activity using different support materials							
	Control		Luffa pulp		Pumice		Ca-alginate beads	
Wild *E. faecium* EP1	IZ(mm)	AU/ml	IZ(mm)	AU/ml	IZ(mm)	AU/ml	IZ(mm)	AU/ml
	16	10.24	13	6.76	17	11.56	12	5.76

IZ = Inhibition Zone (mm) - AU= Activity Unit/ml

the polymerase chain reaction [25,51] to obtain 16S rRNA-specific genes for sequence analysis. Sequencing of PCR-product and comparison of 16S rRNA (rDNA) sequences has been reported among type strains of *Enterococcus* [24,6,51].

The phylogenetic relationships among the new experimental isolate S14 and the closely related *Enterococcus* species have been described in the present work and revealed that, strain S14 was taxonomically positioned within the *E. faecium* group representing 92% identity. The data obtained by 16S rRNA coincide with those found by traditional, morphological, physiological and biochemical methods. This strain was identified as *E. faecium*.

Different type cultures were tested for their sensitivity to the bioactive compounds produced by *E. faecium* to achieve the highest antagonistic activity.

E. faecium was grown in Nutrient agar, ZoBell and LB media and was tested for the antagonistic effect against *S.aureus*. LB was found to be the most suitable medium to achieve the highest antagonistic activity as was reported by [1].

In the present study, Plackett-Burman design was employed which was successfully employed in enzyme production and other optimization experiments [25,51,2]. Results revealed that concentration of yeast is the main factor affecting the activity of the bioactive compounds produced by *E. faecium* where it was significant at 1% level. Decreasing the levels of this factor yielded the highest antagonistic effect against the tested pathogen.

It was noticed that the highest production of the antimicrobial agent by *E. faecium* was obtained at pH 8. [17] reported that pH 8 was the optimum pH for the maximum production of bioactive compounds from *Nocardia brasiliansis*.

Leal–Sánchez, [34]. reported that temperature was found to have positive significant effects ($p \leq 5$) on the production of the bioactive compounds which is in agreement with our study. In the present study, sea water concentration exhibited positive effect on the production by *E. faecium* as shown by [36]. Inoculum size had also positive effect on the production [2].

Another study carried out by [28]. stated that fermentation time is very important optimizing factor. In this study, the maximal production of the bio-active compounds was obtained by *E. faecium* after 48 h after which, the activity decreased significantly in the culture medium. Similar results were also reported by [10].

It was shown that increasing the incubation period, peptone, inoculum size, temperature, pH and sea water concentration have positive effects on the production of bioactive compounds by *E. faecium*. Therefore, results concluded that to achieve the highest antagonistic effect by *E. faecium,* the medium composition should be: (g/l) peptone, 15; yeast extract, 2.5; sea water concentration (<100%), pH 8 with inoculum size 1.5 ml (for each 50 ml medium) for 48 hrs at 35°C. Under such conditions, the activity unit produced by *E. faecium* showed 10.24 AU/ml (2.56 fold increase) than that obtained using the basal growth medium.

The effect of mutations on the production by different bacterial strains have been reported (Hosoya *et al*., 1998) but in our study, the mutations decreased the production of bioactive compounds.

Immobilization on pumice showed (1.13) fold increase in the productivity compared to the free cells while immobilization on luffa and inside the beads of sodium alginate reduces the productivity and these results may be due to one or more of the following reasons: the support may possesses poor mechanical stability, as reported by Klooster and Lilly, [32]. Diffusion limitation is a second important factor where substrate limitation/product inhibition in entrapped immobilization systems may also affect this process [5]. A third factor is the individual characteristics of the bacterium [31]. where Ivanova *et al*., 1998 stated that the antibiotic production of 12 strains of epibiotic bacteria was enhanced after immobilization.

We can conclude that marine bacteria *E. faecium* have the potentiality to produce higly effective bioactive compoundes which must be applied in aquaculture or in the production of pharmacutical agents.

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