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Production of Protease by *Enterococcus faecium* E745 and *Enterococcus faecium* PSB 5 isolated from protein rich soils

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ABSTRACT

This study was focused on the isolation and identification of some bacterial strains producing protease. For this purpose, a then 10 samples of protein rich soils from dairy factories, cattle's feeding factories, and slaughterhouses, were collected from the region of Chief, located in Northwest Algeria, where the wastes are submitted to natural fermentation. The primary screening has been achieved on agar milk culture medium and the selection of the producing bacterial strains was based on the determination of the produced diameter of hydrolysis zone on the surface. The obtained results indicated the isolation 115 bacterial strains belonging to different genus. Furthermore, the 4selected protease producing strains were identified by the study of their biochemical characteristics, the amplification of the fragment ADN, coding the region of 16S ARN and their sequencing. The analyze of the amplified, sequenced DNA fragment, coding the region of 16S ARN fragments of the 4 selected protease producing strains by the using of RDP Project and BLAST), indicated their identification as *Enterococcus faecium* E745 and *Enterococcus faecium* PSB 5, respectively. Furthermore, the using of SDS-PAGE for protein profile indicated the presence protein bands around 35-37 KDa.

Keywords: Protease, Isolation, Protein-rich soils, 16S rDNA, *Enterococcus faecium*, *Enterococcus faecalis*.

INTRODUCTION

Proteases are the most important class of industrial enzymes, which that secrete a wide molecules variety with several functions and implicated in various important biotechnological applications [1]. Furthermore, proteases described as single class of enzymes occupied a pivotal position by the application in both physiological and commercial fields with about 60 % of total worldwide enzymes market [1]. Proteases enzyme employed in several areas such as pharmaceutical, food and detergent industries [2-4]. The available commercial proteases mainly produced by microbiological process, animal, and plant sources [3, 4].

Furthermore, proteases have been produced by several fungi, yeast and bacteria such as *Bacillus sp*, *Alcaligenes faecalis*, *Pseudomonas fluorescens*, *Aeromonas hydrophilia*, and *Enterococcus sp*, cultivated under submerged culture conditions [4, 5, 6]. However, the produced proteases by *Enterococcus faecium* have a considerable importance due their biochemical character and their wide application in dairy industries including food industries, their use for acidification of the milk, the development of flavor, for their antimicrobial activity and their strong proteolytic activity. Furthermore, the availability of genomic DNA of *Enterococcus faecium*, exempted from any virulence factors [7, 8], and the presence of the highest protease activity may be used as the source of wide applications in the field of health [9]. Therefore, the main aim of the present work was the isolation of a new proteases producing strains with novel properties, the determination of their biochemical characteristic and the molecular identification of the isolated, selected protease producing *Enterococcus* by the amplification of the gene, coding the region of 16S ARN and their sequencing.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from dairy factories, cattle's feeding factories, slaughterhouses, by the using of a sterile spatula, where the first 5 cm of the upper layer of the soil, composed essentially of the degraded protein wastes were removed, and 100 g of soil was collected from the depth between 5-15 cm. The samples were placed into a sterile plastic bag, transported to the laboratory, and kept at temperature of 4°C [11, 12].

Isolation of bacterial strains from soil samples

1 g of fresh weight of soil sample was aseptically added to a volume of 9 ml of sterile distilled water, homogenized on a shaker for a period of 45 min. For isolation of producing protease bacteria strains [7], a volume of 0,1 ml of each sample, prepared by serial dilution (up to 10^{-6}), was aseptically spread on skim milk agar plates, which was composed of the following components : peptone (0.1%), NaCl (0.5%), skim milk (10%), incubated at temperature of 30°C for 24 hours. The protease activity of the isolated, bacterial strains was assayed by appearance of clear zones around the inoculated bacterial colonies on the surface of the solid culture medium. The selected producing protease colonies (T_2 , T_2) were purified on the early used solid culture medium and investigated for their morphological and biochemical characteristics [13].

Biochemical characterization

The use of biochemical characters such as Gram's reaction, catalase activity, motility test, the presence positive reaction of bile-esculin, salt tolerance tests and the cultivation on blood agar culture medium were determined according to the standard procedures described by Gross and co-workers (1975) [13, 17].

Genomic DNA extraction

The bacterial strains were grown in nutrient broth, incubated on shaker at 120 rpm at temperature of 30°C for overnight. The genomic DNA was extracted according to the method described by Sambrook and Russel (2001) with a minor modification. For

this purpose, a volume of 1,5 ml of overnight culture broth was harvested by centrifugation at 5000 rpm for 10 min and the supernatant was discarded. The recuperated pellet was dissolved in a volume of 200 µl of lysis buffer (200 µl of triton 200 % ,1000 µl of SDS 1%, 200 µl NaOH at 100 mM, 100 µl of Tris at 10 Mm, 20 µl EDTA, 6,68 ml H₂O), added 0,3g of glass beads. After that a volume of 200 µl of solution containing the following components (phenol: chloroform: isoamyl alcohol) was added, homogenized and centrifuged at 13000 rpm at temperature of 4°C for 5 min. The liquid phase was transferred in new Eppendorf tube and a volume of 20 µl of 3M sodium acetate, adjusted at pH-value of 4.8, was added. A volume of 600 µl of ethanol was added to the mixed solution, homogenized and kept at temperature of -20°C for one hour [18]. The supernatant was discarded and the pellet was washed with 70% ethanol and the genomic DNA was harvested by centrifugation at 13000 rpm for 5 min, conserved at temperature of -20°C. The genomic DNA was dried at ambient temperature for 10 min and dissolved in a volume of 50 µl of sterile distilled water and kept at ambient temperature for 30 min [18, 19]. After that, a volume of 1 ul of RNase solution was added to the extracted genomic DNA, incubated at temperature of 37°C for 20 min.

Amplification of 16S rDNA gene by polymerase chain reaction

A universal primer was used for the amplification of DNA fragment, coding the region of 16S rDNA of the 4 selected bacterial isolates. For this purpose, a volume of 50 µl of reaction mixture containing 100 ng of genomic DNA, 0.25 µl taq polymerase (5U/µl) ,5 µl of 10X buffer 5 prime, 5µl dNTP 10 Mm, and 0.5µl of primer [19, 20]. The used forward primer and reverse primer has the following nucleotide **W001** ('5-AGA GTT TGA TCM TGG CTC- '3), **W002** ('5-GNT ACC TTG TTA CGA CTT-'3) respectively. The amplification of the targeted DNA fragment was carried out according the following polymerase chain reaction (PCR) by the application of the followed program conditions: The reaction mixture was consisted of an initial denaturation step for 4 min at 96°C, followed by 30 cycles of denaturation for 10 sec at 96°C, annealing at 50°C for 30 sec, and final elongation at 72°C for 2 min [18]. The amplified polymerase chain reaction (PCR) products (5 µl) were analyzed by electrophoresis in 1,2 % agarose gels stained with ethidium bromide. (1 Kb ladder invitrogen). Furthermore, the product was purified by the using PCR kit (Nucleo Spin Gel and PCR Claen Up) and sequenced according the method described by Kunitz [18].

DNA sequencing

The sequencing of 16S rDNA was carried out at Beckman Coulter Genomics, Takeley, CM22 6TA Essex United Kingdom. The sequenced 16S rDNA was analyzed with nucleotide database available at the GenBank using RDP project and Blast tool at NCBI for identification of bacteria and were submitted at NCBI Gen Bank [18].

Enzyme purification

The total protein purification procedure was performed at temperature of 4°C. A volume of 5µl of the overnight culture of the isolated selected protease producing bacterial strains was harvested by centrifugation at 4000 rpm for 5 min. The biomass of the cell culture was dissolved in a volume of 500 µl of lysis buffer (0,5 M LSDB), dialyzed against glass beads, for 2 min in vortex. The recuperated dialysate was concentrated by centrifugation at 14000 rpm for 10 min. After that, a volume of 100 ml of the blue SDS 2X was added to the concentrate. The protein content was measured by the using bovine serum albumin as standard, according to the described protocol by Bradford [19].

SDS-PAGE

Precast polyacrilamide gel (NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels) was used for optimal separation of protein under denaturation conditions according to the described method by Meunier [21]. For this purpose, a volume of 119 µl of the

prepared simple aliquot of protein crude extract of both isolated selected bacterial strains (T_2 , and T_2 op) was separately added to a volume 40 μ l of the blue solution respectively [19]. Samples were denatured at temperature of 95°C for 5 min and the molecular weight was estimated by using bovine serum albumin as standard [20]. The samples were applied in Laemmli buffer (900 μ l Laemmli sample buffer Biorad, 4X, 100 μ l of 2- Mercaptoethanol) run at 160 V for one hour. The SDS solution was removed by the twice rinsing of the gel for 30 minutes on shaker at 50 rmp and the staining with Coomassie Brilliant Blue R-250 and detained with a solution containing the following components (methanol 20%, acetic acid 10%), the appeared band protein was explored by the using of ultra violet lamp [21].

RESULTS AND DISCUSSION

Isolation of protease producing bacteria

The achieved primary screening was allowed the isolation of 115 bacterial strains from 10 samples of soils such as dairy factories, cattle's feeding factories, and slaughterhouses, which were very rich in proteins and manifested high protease activity. The secondary screening was based on the protease activity on the agar milk culture medium (GN/TGEA), containing skim milk at a concentration of 10%, has revealed the selection of 4 proteases producing bacterial strains (T_2 , T_2 op, NB_1 op, and Y_4), with hydrolysis zones. The obtained results according the formed hydrolysis halos presented in (Figure 1), indicated that both selected protease producing bacterial strains (T_2 , T_2 op) manifested the formation of the highest halo of hydrolysis zone with diameters of (38 and 36 mm) respectively. Whereas, the two others selected isolates (NB_1 op and Y_4) has manifested a halo of hydrolysis zone with diameters of (32 and 30 mm). Since, the diversification between the selected protease producing bacterial strains was based only on the sample origin (soil) and environmental conditions, the protease activity variation between the strains was not the consequence for species variability. Whereas, the maximum protease activity was recorded after bacterial strains adaptation. Wang and co-workers have reported that a maximum protease activity was observed after 48 hours' incubation [23, 24],

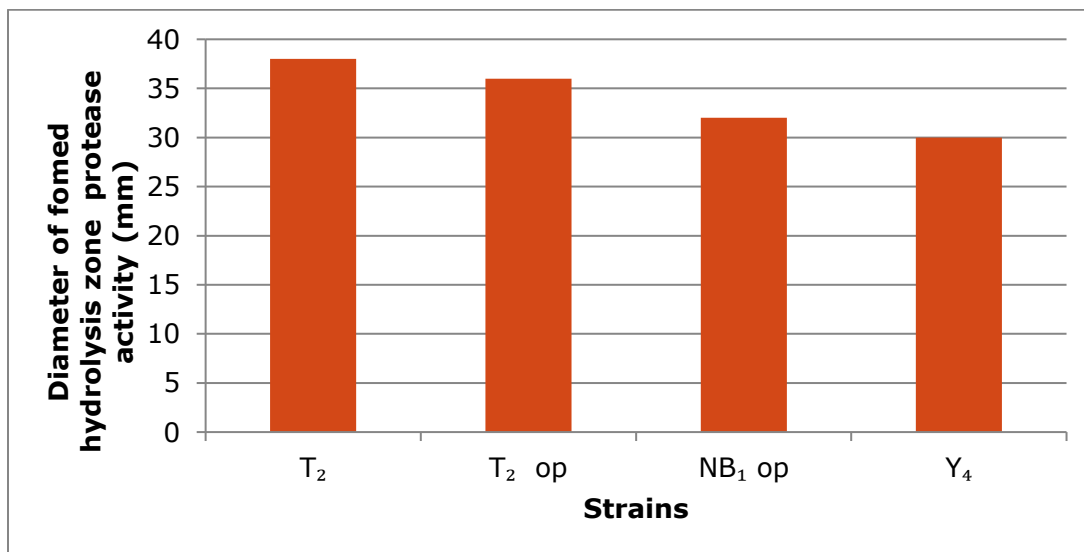


Figure 1: Illustration of Protease activity of the isolated, selected bacterial strains (T_2 , T_2 op, NB_1 op, and Y_4), inoculated on solid culture medium, incubated at temperature.

Morphological and biochemical characterization of the isolated, selected bacterial strains

The morphological study, the microscopic observation, and the study of the biochemical characteristics such as catalase activity, motility test, culture on blood agar culture medium, and shape revealed that the isolated, selected bacterial strains (T_2 , T_2 op,

NB₁ op, and Y₄) belongs to the genus *Enterococcus* spp. Furthermore, the microscopic observation and the biochemical characteristics of the isolated, selected bacterial strains showed as Gram positive, ovoid or spherical cocci, anaerobic, catalase negative and spore non-forming. The tests more commonly used to identify gram positive bacteria as members of the genus *Enterococcus* are listed in Table 1 [17]. The presented morphological, physiological and biochemical aspect of the isolated selected protease producing bacterial strains (T₂, T₂ op, NB₁ op, and Y₄) in Table 1 indicated their membership to the genus of *Enterococcus*. Furthermore, the cultivation of the isolated selected protease producing bacterial strains (T₂, T₂ op, NB₁ op, and Y₄) on the solid culture medium indicated the presence of smooth, cream or white colonies. Whereas, the cultivated *Enterococcus faecalis* on the culture medium sheep blood agars was non-hemolytic and beta-hemolytic on culture medium containing rabbit, horse and human blood. Whereas, *Enterococcus faecium* was alpha-hemolytic as described by Bergey's Manual of Determinative Bacteriology [22].

Table-1: Presentation of morphological, physiological, and biochemical aspect of the isolated selected protease producing bacterial strains belonging to the genus of *Enterococcus*.

Investigated test	% Positive reactions of strains of the genus
	<i>Enterococcus. sp</i>
Gram-positive cocci	100
Group D antigen	77
Bile-esculin reaction (40% bile)	100
Growth in 6.5% NaCl broth	100
Gas from glucose	99
Vancomycin susceptibility	>1

Selection of protease producing strains

The use of the phenotypic character for identification of the isolated, selected protease producing bacterial strains (T₂ and T₂ op) and the study of hydrolyses of casein indicated the memberships of the isolates to the genus *Enterococcus* (Figure 2). Furthermore, the bacterial growth of both isolated, selected protease producing bacterial strains (T₂ and T₂ op) was observed on both used culture media such as milk agar and skim milk agar in the presence of a concentration of (10%) Figure (3, 4).

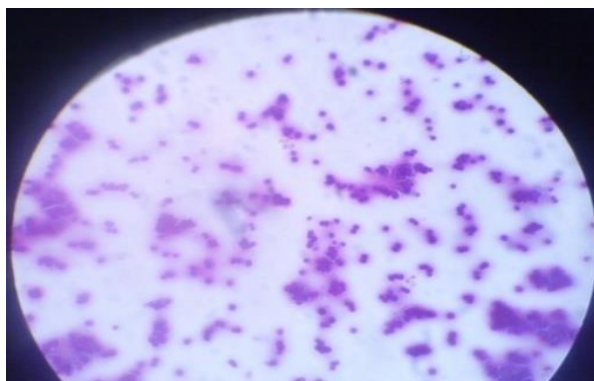


Figure-2: Microscopic observation 100 × selected protease producing *Enterococcus faecium*, showing cocci shape and Gram positive and spore non-forming Bars = 10µm.

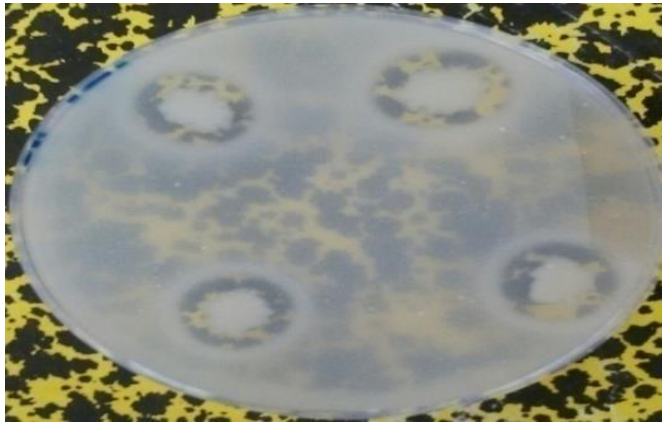


Figure-3: Protease activity of the selected *Enterococcus faecium* on the skim milk agar culture medium (T₂).

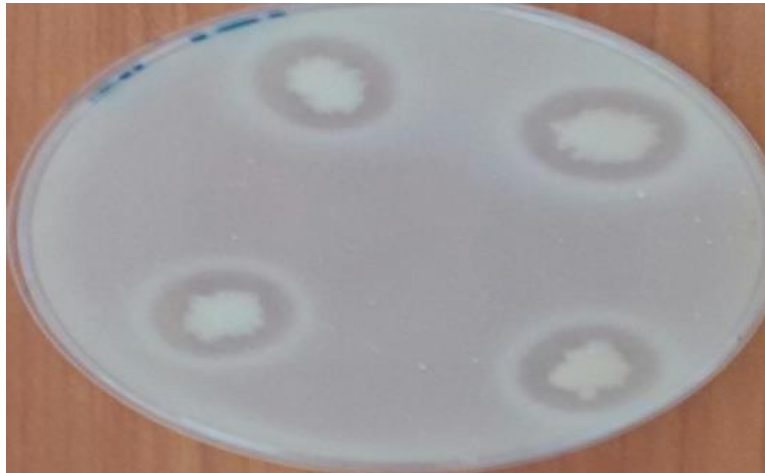


Figure-4: Protease activity of the selected *Enterococcus faecium* on the skim milk agar medium (T₂ op).

Molecular identification of strains

The 16S rDNA genes of the isolated, selected bacterial strains (T₂, T₂ op, NB₁ op, and Y₄) were amplified with success and visualized by the using of agarose gel electrophoresis (Figure 5). The amplified obtained product revealed the presence of bands of 1000 bp, which was the size of the target fragment.

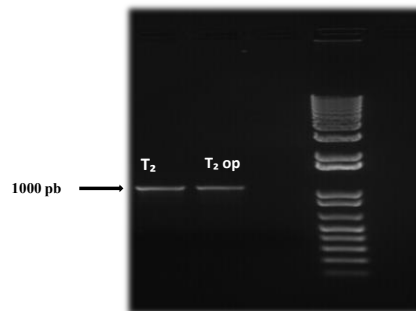


Figure-5: Agarose gel electrophoresis of the amplified 16S rDNA gene of the the isolated, selected bacterial strains (T₂, T₂ op).

Based on 16S rRNA gene analysis, the isolated, selected bacterial strains (T₂, T₂ op, NB₁ op, and Y₄) were phylogenetically characterized and identified by the using of NCBI database with BLAST, which confirmed their memberships to *Enterococcaceae* family by the using RDP project (Figure 6). Furthermore, the use of the homology search of 16S rDNA nucleotide sequence indicated the presence of high identity to the recorded *Enterococcus* strains in the NCBI data base (Table 2).

Table-2: Strains 16S rDNA identification

Isolate code	Strain Homology	identification	Accession number
T ₂	<i>E. faecium</i> strain E745	<i>E. faecium</i>	CP014529.1
T ₂ op	<i>E. faecium</i> strain PSB 5 16S	<i>E. faecium</i>	AB607166.1
NB ₁ op	<i>E. faecalis</i>	<i>E. faecalis</i>	AY395018.1
Y ₄	<i>E. faecalis</i> strain RCB443	<i>E. faecalis</i>	KT260655.1

The construction of phylogenetic tree by the using of the obtained 16S rDNA sequences and the sequences of other strains has been achieved for the comparison of the taxonomic position of our isolated strains was performed (Figure 6). Where, the analysis of 16S rDNA sequence of the isolated, selected bacterial strains (T₂, T₂ op, NB₁ op, and Y₄) submitted in the GenBank database and the study of the alignment of these sequences by the using BLAST showed the presence of 99% sequence similarity with *Enterococcus faecium* E745, 99% sequence similarity with *E. faecium* PSB5, 99% sequence similarity with *E. faecalis* and 99% sequence similarity with *E. faecalis* strain RCB443 as the closest species. Furthermore, the study of Dendogram showed that both strains *Enterococcus faecium* E745, and *Enterococcus faecium* PSB5 were closely related with 60% similarity and (100% to *Enterococcus faecium* DO. Whereas, *Enterococcus faecium* E39 presented (82% similarity) to *Enterococcus faecium* UW8175 (82% similarity), which are most used in dairy industries due to their strong proteolytic potential. However the strains were farther from *Enterococcus* pathogenic species such as *E. faecalis* and *E. faecalis* RCB 443 (87% Similarity).

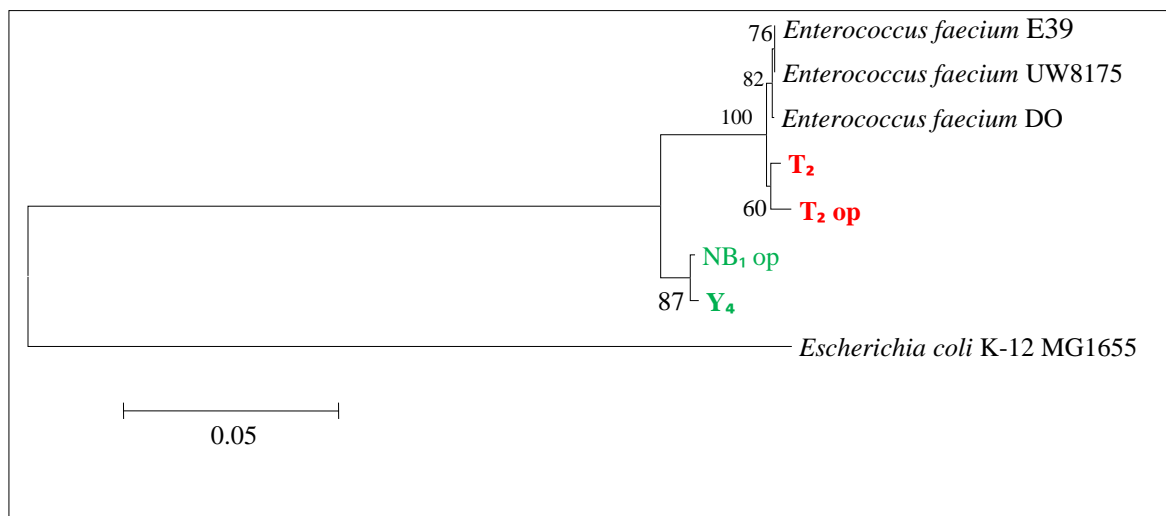


Figure-6: Phylogenetic tree based on sequence analysis of 16S rDNA gene using the Neighbour-joining method. and showing the relationships between the strains (T₂, T₂ op, NB₁ op, Y₄) and the other similar species.

SDS-PAGE analysis of proteases

SDS-PAGE method was used for the exploration of the homogeneity and molecular weight of proteases produced by bacterial strains [25, 26]. Different protein bands corresponding to molecular weights ranging 35-37 KDa was recorded the separation of the protein containing in the crude extract of *Enterococcus faecium*, which showed only one band within the gel electrophoresis suggested as purified protein was homogeneous (Figure 7) [27]. The results were similar to the reported results by Macwana et al [28], where the molecular weight of purified and dialyzed protein was 23 kDa.

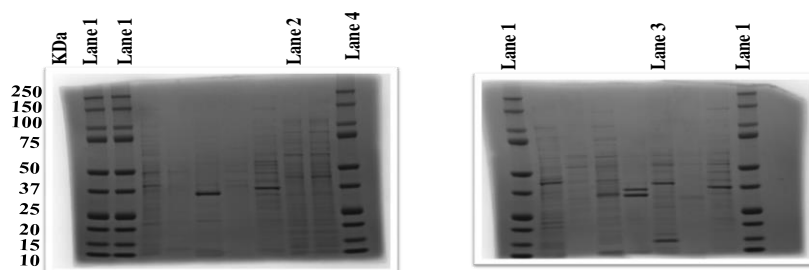


Figure-7: Photographic representation of protein profile of *E. faecium* strains (Lane1, Protein marker; Lane 2, Protein from T₂; Lane 3, protein from T₂ op; Lane 4, Bleu1X).

The results indicate differences in the sizes and the number of protein bands characteristic of each strain. This shows that the two strains having different protease activities.

DISCUSSION

Enterococci was the memberships of the lactic acid bacteria (LAB), which have ambiguous significance in foods with both beneficial and detrimental aspects [29]. They have controversial properties such as opportunistic pathogenic, where are able to induce human infections [30]. On the other hand, *Enterococci* are used for a maturation of various raw milk cheeses and the

inhibition pathogen bacteria growing in food [30, 31]. The aim of the present work was the isolation of a new proteases producing strains with novel properties, the determination of their biochemical characteristic and the molecular identification of the isolated, selected protease producing *Enterococcus* by the amplification of the gene, coding the region of 16S ARN and their sequencing.

The microscopic observation and the biochemical characterization showed the isolated, selected protease producing enterococci strains as Gram positive, ovoid or spherical cocci, anaerobic, catalase negative and spore non-forming. Furthermore, the cultivation of the isolated selected protease producing bacterial strains (T₂, T₂ op, NB₁ op, and Y₄) on the solid culture medium indicated the presence of smooth, cream or white colonies. Whereas, the cultivated *Enterococcus faecalis* on the culture medium sheep blood agars was non-hemolytic and beta-hemolytic on culture medium containing rabbit, horse, and human blood. Whereas, *Enterococcus faecium* was alpha-hemolytic as described by Bergey's Manual of Determinative Bacteriology [22]. Moreover, the identification of the isolated, selected protease producing strains by the using of PCR analysis, where the amplified 16S rDNA nucleotide was sequenced by automated sequencer.

The nucleotide sequence of the of the isolated, selected protease producing strains (T₂ and T₂ op) was compared with data/bank at NCBI by blast and showed highest similarity (99%) with the strain *Enterococcus faecium*, Nucleotide accession number for strains is respectively CP014529.1 for *E. faecium* E745 and KM095647.1 for *E. faecium* PSB5. To characterize the isolated, selected protease producing *Enterococcal* strains the SDS-PAGE of the crude extract protein has been achieved according to the described method by Dupre [32] and the protein profiles were compared with the reference of the available collection strains. The protease producing *E. faecium* isolates showed a similar protein profile by the using of SDS-PAGE, which the PCR products were observed for both isolated selected protease producing species [7]. Moreover, the obtained results indicated that both protease producing strains *Enterococcus faecium* E745 and *Enterococcus faecium* PSB 5 has manifested different bands protease. The distribution of the selected genes, which were responsible for the virulence of the enterococcal bacterial strains was studied for the exploration of theirthe pathogenic potential in the environmental. The achieved early studies on Tunisian artisan fermented meat products, Mukesh [8] have reported that 10 of 24 *Enterococcus faecium* protease producing bacterial strains were denied from any virulence factors [8]. Whereas, the virulence genes *cylA*, *gelE* and *esp* coding for hemolytic cytolysin, gelatinase and enterococcal surface protein, respectively monitored by 61 bacterial isolates were illustrated only in four strains of *E. faecalis* [33, 34].

The published results belong to the few investigation for the determination of the virulence genes distribution within environmental enterococci, which suggested that *E. faecalis* was the species with more virulence genes than other enterococci [7, 8, 33, 34]. Furthermore, the characterization of the isolated, selected protease producing bacterial strains involved the detection of genes, which were coding the expression of the enterocin small secreted peptides, responsible for antibacterial activity. In early studies, Ennahar and Giraffa [35-37] have reported that the produced enterocin by *E. faecium* was the only factor responsible for antibacterial activity, which was employed in food industry as probiotic for the inhibition of borne pathogens [38]. The obtained results suggested that the used *Enterococcus faecium* E745 and *Enterococcus faecium* PSB5 protease producing, devoid of virulence factors may have various biotechnological interests. In conclusion, the members of the genus *Enterococcus* posed a serious difficult for their identification on species level by the use of a unique identification system. Therefore, the use of useful biochemical tests is for a preliminary identification coupled with the molecular methods was required for reliable results [39]. The employed PCR methods are the most promising and the most reliable, although in some cases are unable to identify the isolates well [7, 39]. The primary PCR screening revealed again the presence of the various useful genes by *Enterococcal* strains and their the ambiguous nature, which were used as probiotic bacteria [39]. However, the isolated, selected producing proteases of *Enterococcus faecium* have a wide industrial application in dairy industries including food industries due their biochemical

character, their role by the acidification of milk, the development of flavor and their antimicrobial activity against such pathogenic bacteria [9].

CONCLUSION

Two protease hyper producer strains were isolated and identified as *Enterococcus faecium* from protein rich soils. These strains were close to a group of lactic bacteria used in dairy industries and particular food industries; therefore, they could be valued as an excellent agent of fermentation process. Also, proteases of *Enterococcus faecium* have a considerable industrial potential. The protein profile in SDS-PAGE revealed protein bands around 35-37 KDa. To conclude, the protease from *Enterococcus faecium* may be further exploited in various biotechnological applications in large scale.

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