Interspecific Protoplast Fusion Between *Streptococcus lactis* Rennin producing Strains

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**Abstract:** In attempts to construct superior *S. lactis* strains producing rennin enzyme, protoplast fusion was done between *S.lactis* T6 and *S.lactis* T4 animal rennin producing and *S.lactis* T3 rennin–like producing. Eight fusants were obtained and their enzymatic activity was determined. The milk clotting activity (M.C.A.) and proteolytic activity (P.A) of *S.lactis* rennin and rennin-like producing strains and their fusants were evaluated. All fusants were characterized on the bases of M.C.A and P.A. The parent *S.lactis* T6 rennin producing showed sharp increase of the coagulation time, and their fusants, F-2, showed high M.C.A, 0.985, also F-4 showed lower proteolytic activity than their parents. Electrophoretic protein profile, SDS-PAGE, of these fusants and their parents were studied and the relatedness between them was determined according to their Phylogenetic tree.

**Keywords:** Chymosin, Proteolytic activity, Milk-clotting activity, and SDS-PAGE, *Streptococcus lactis*

**INTRODUCTION**

The lactic streptococci are widely used for dairy and other food fermentations\[^9\]. Recent reviews emphasize the importance of gene transfer mechanisms in studying the molecular biology of these bacteria and in constructing new or improved strains via recombinant DNA technology\[^9,11\]. One of these mechanisms, protoplast fusion, has been used for the transfer of both plasmid and chromosomal genes between genetically marked derivatives of lactic *Streptococcus*\[^8,13\] and for the intergeneric transfer of a plasmid from Streptococcus lactis to *Lactobacillus reuteri*\[^4\].

Molecular genetics of dairy lactic acid bacteria, including development of techniques of gene transfer, has advanced rapidly in recent years. Protoplast fusion is one of the most promising methods of gene transfer in both genetically studies and improvement of starter strains for practical use in dairy industries. Recently\[^8,10\] investigated the recombination of chromosomal markers and transfer of plasmid markers (*lac* and *ery*) by protoplast fusion, and showed that the frequency of colonies appearing on selective media was increased 10 to105 fold when the mixed protoplasts from the two parent strains were treated with polyethylene glycol(PEG)1000.

The work within hand aims at improving the chymosin production via genetic construction, using protoplast fusion technique, of *S. lactis* rennin producing enzyme strains obtained from the author. Also, the use of protoplast fusion for the intergeneric transfer of chymosin gene between *S. lactis* strains which producing rennin and rennin like enzyme was described.

**MATERIALS AND METHODS**

Three *S.lactis* strains were used through this investigation. The characters and the source of each one are presented in Table (1) as follows:

**Media Used:**
- Luria-Bertani medium (LB) was used as a complete medium for propagation and stock culture of *S.lactis*\[^5\].
- (Cheeseman, \[^3\] this medium was used for isolation of rennin producer strains.
- M17-gl medium was used for *S.lactis* protoplast experiments, \[^15\]
- 2% agar was added to the previous broth media in order to obtain solid media. All media were sterilized by autoclaving at 121°C for 20 minutes under pressure of 15lb/square inch.
- Protoplast buffers were used according to Simon *et al.*\[^16\].
- Formations of protoplast was performed according to Simon *et al.*\[^16\] and Kondo and McKay\[^12\].

**Isolation of Rennin Producer Fusants:** All fusants have been screened for rennin production following the method adapted by Ganguli and Bhalaria\[^7\] which carried out by plating appropriated dilution of bacterial
cells in nutrient agar medium supplemented with 1% soluble casein and 0.11% CaCl₂. In this medium rennin-producing strains show rennin specific zones, precipitated zone, while proteolytic enzyme producer, a clear zone. The plates were incubated at 30°C.

-SDS-PAGE was performed by the method described by Sheri et al.[17].

RESULTS AND DISCUSSIONS

Protoplast Fusion: Protoplast fusion technique was hybrids of two or more species, which could not be obtained by classical mating. Polyethylene glycol (PEG) was an effective and powerful fusogenic agent. Inter-generic or inter-specific fusion provides a method for the introduction and transfer of desirable genes e.g., the lactose permease and β-galactosidase genes[2].

Strains *S. lactis* T6, T4 and T3 were chosen for genetic improvement attempt via protoplast fusion. The primary step was the estimation of parents’ strains capability in protoplast induction by enzymatic removal of the cell wall. The cells were incubated at 37°C for 3 hr with lysozyme and α-amylase (300 µg/ml) in SMMB buffer[6]. The results are presented in Table (2).

Protoplast Induction: Table (1) presents *S.lactis* strains sued and their indentifying characters. *S.lactis* T6 was chosen as a common parent with strains *S.lactis* T4 and *S.lactis* T3 essentially because it is highly produce rennin enzyme only whereas the other strains produce rennin with pepsin and rennin like enzyme, respectively [1]. This chosen design may increase the probability of increasing the gene dosage of rennin in fusants.

Protoplast Induction and Regeneration Efficiency: Lysozyme and α-amylase (300 µg/ml) was added to the treated *S.lactis* cells and incubated at 37°C for 3 hr at 40rpm. Protoplast induction was followed periodically via microscopic examination; almost all enzymatic treated cells were converted into protoplast within 3 hr as revealed via microscopic examination.

In order to determine the efficiency of protoplast induction and regeneration for each parent strain two equal samples of pretreated cells, protoplast suspension, were used. The first was spread onto M17-glc medium after osmotic shock and the number of intact cells was counted (Table 2). The second part was added to the top layer medium and overlaid on the regeneration medium and grown cells were also counted. From these results the number of regenerated protoplasts and efficiency of protoplast induction for each parent were calculated (Table 2).

Protoplast Induction and Regeneration: The only parameter examined which significantly affected regeneration frequency was the method of plating protoplasts. We performed regeneration studies by exposing protoplasts to a 15-min PEG treatment followed by dilution, centrifugation, and suspension as described above for transformation. Since soft agar overlays were shown to enhance the frequency of protoplast regeneration, they were used in transformation experiments to determine if transformation variability could be accounted for by this parameter. The highest protoplast induction reached 76.54% for the parent *S.lactis* T6, while it was and 55.38 for *S.lactis* T4 and 48.71 for *S.lactis* T3. This result presented in Table (2) is considered as positive factor in addition to the previously mentioned reasons for choosing strain *S. lactis* T6 as a parent in common to increase the probability of fusants induction.

Inter-generic protoplast fusion. Between *S.lactis* T6, *S.lactis* T4 and *S.lactis* T3.

The chosen two parents; *S.lactis*T6 and *S.lactis*T4, have distinctive antibiotic markers which beneficial in fusants selection illustrated in Table (1). In order to select fusants between parents. The protoplast suspension was mixed in presence of PEG 25% and 100 mM CaCl₂. After 60 min of incubation at 30°C, 100ml, and sample was added to M17-glc medium 1% soluble casein supplemented with; Cm, Te, Rif. Cells that resisted of antibiotics were selected as fusants cells. In another attempt to induce inter-generic fusants two parents, *S.lactis* T6, animal rennin producing, *S.lactis* T3, rennin like producing, were used and their characters were shown in table (1).cells that resisted both Tc and Nm antibiotics were selected as fusants.
Results presented in Table (3 and 4) proved the success of fusants formation between the three parents.

**The Milk Clotting Activity and Proteolytic Activity:**
The inter-generic fusion products were tested for their milk clotting capability. The milk clotting activity and proteolytic activity were done for the three rennin producing transformants; *S. lactis* T4, *S. lactis* T6 and rennin-like enzyme *S. lactis* T3 and their of fusants (Table 3 & 4). In the trial of *S. lactis* T6::*S. lactis* T4 all obtained, genetically stable, five fusants were showed proteolytic activity less than the parent *S.lactis* T4 which has proteolytic activity 0.842U/ml. Three fusants out of them; *S.lactis* 2,4 and 5 reached high Milk clotting activity over proteolytic activity ratio, which gave ratio of 56.28%, 76.11% and 3.26%, respectively than the other parent, *S.lactis* T6, which reached 33.10%, and also showed lower proteolytic activity than the two parents (Table 3). Only one fusants, *F4*, Table (3) showed low proteolytic in comparison with the two parents and other fusants. Also, one fusants, *F2*, Table (3) showed more M.C.A than the parent *S. lactis* T6. Data present in table (3) showed that all the fusants have lower Milk clotting activity than the parents and all the obtained fusants, genetically stable, have a good curd formation.

Also data in Table (4) showed that the parent, *S.lactis* T3, has high proteolytic activity and low milk clotting, M.C.A/P.A ratio in comparison with the *S.lactis* T6 parent as I reached the higher rate of proteolytic activity,0.680, than the other parent *S. lactis* T6. The three fusants, *F1*, *F2* and *F3*, showed lower P.A than their parents and also M.C.A, they reached the lower M.C.A than parent *S.lactis* T6. These results inagreement with the results obtained by Attallah et al., (2007) they found that all *S.lactis* renmin and rennin like producing strains showed high M.C.A/P.A ratio. The fusants obtained were isolated following the method adopted by Cheeseman[3] and their cell-free cultures were tested
by casein agar plate method[7]. No difference in the capacity of enzyme production was detected; i.e. no differences in diameters of the specific zone which formed around tested fusants.

**Protein Electrophoresis:** Electrophoretic separation based on SDS-PAGE of cell-free extract or of cell wall proteins was used for the identification and/or differentiation of different microorganisms also, used for typing certain bacteria[14]. In this investigation, SDS-PAGE, was used for the differentiation of different *S. lactis* rennin and rennin producing and their fusants.

Gel was scanned and analyzed using Quantity one D (Bio-Rad). The SDS-PAGE photograph is given in Fig (1). In this prospect the SDS-PAGE was successfully used for the differentiation of *S. lactis* animal rennin and rennin like producing and their fusants. The analysis of whole cell protein extract of eight fusants and their parents by one Dimensional SDS-PAGE revealed a set of clearly different profiles. Gel documentation system, image analysis software, was used for more accurate for their analysis and comparison between the parents and fusants obtained in inter generic protoplast fusion pre-mentioned. Fig. (2)
shows result of gel-documentation for all strains used in protoplast fusion through this study and their fusants depending on protein profile and migration on SDS-PAGE.

Phylogenetic analysis for the protein profiles of the parent \textit{S. lactis} T6 with \textit{S. lactis} T4 and \textit{S. lactis} T3 and their fusants from one to three resulted in the tree which shown in Fig. (2b) and with other the rest fusants, (Fig. 2a). The tree which shown in fig. (2b) evidenced that fusants; F-3, F-4, and F-5 are cluster arranged in descending manner according to their relatedness to the parent \textit{S. lactis} T6 and parent \textit{S. lactis} T3, while for the other cluster tree fig. (2a) fusants, F-4, F-5, F-6 and F-7, are arranged in descending manner according to parent \textit{S. lactis} T6. Similarity analysis for the protein profiles of the parent \textit{S. lactis} T6 with \textit{S. lactis} T4 and fusants from one to five resulted in the tree which shown in fig.(2a).

In Conclusion, The success of fusants formation between \textit{S. lactis} strains rennin and rennin like producing. The success of protoplast fusion in \textit{S. lactis} was proved to develop new genetically stable strain with high efficiency of rennin enzyme production. It is recommended the genetic improvement must be doing on the high efficiency fusants increase its efficiency or to obtain a new strains harboring desirable economical characters concerning rennin induction.

REFERENCES