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Fermentation of Milk Permeate by Proteolytic Bacteria for Protease Production

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ABSTRACT

Kinetics of cell growth and protease production by four proteolytic bacterial strains, namely, *Bacillus subtilis* EMCC 1020, *Bacillus megaterium* EMCC 1057, *Serratia marcescens* EMCC 1247, and *Pseudomonas fluorescens* EMCC 1221, in milk permeate, compared with other fermentation media, were studied. The pH values, lactose utilization, and biochemical oxygen demand (BOD) reduction in milk permeate also were investigated. The four strains were able to grow in milk permeate, and to produce considerable amounts of protease, reaching 289 (EMCC 1020), 252 (EMCC 1057), 263 (EMCC 1247), and 212 (EMCC 1221) U/mL after 30 h of fermentation. The growth and enzyme activity of the four strains were greater in milk permeate than those in other fermentation media. The protease-producing bacteria were able to utilize lactose in milk permeate with values ranging from 37.62 to 54.97% and to reduce the BOD of milk permeate by 50.59–63.65%. Milk permeate proved to be the best medium for enzyme production by all organisms examined.

Index Entries: Protease; proteolytic bacteria; milk permeate; BOD; lactose utilization.

INTRODUCTION

Whey obtained from cheese making and casein production traditionally has been considered as a byproduct. Adding to the problem are the large amounts of milk permeate (MP; the byproduct from ultrafiltering milk) now coming on-stream. Whey and MP are highly contaminating

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materials, containing about 35,000 mg/L biochemical oxygen demand (BOD) and 68,000 mg/L chemical oxygen demand (COD), because of the organic matter content, which is ~50% of the total nutrients in the original milk (*1,2*). In many countries, utilization and/or disposing of whey is still a serious problem. Cheese production is increasing worldwide, adding to the complication of whey utilization programs (*3*). Disposal of cheese whey and MP are a major problem for dairy industries. Most (90% or more) MP produced in Egypt was dumped into sewers. The only use of MP is to fill soft cheese containers, which hold the cheese for aging.

Sweet and acid whey or MP have been used as fermentation medium to produce ethanol, (*4*) biomass, (*5*) polysaccharide, (*6*) and ammoniated organic acid (*7*). Of course, these options are challenged by important and variable economic constraints.

Currently, the most important industrial enzymes are the proteases, which are used primarily in the detergent and food industries. Other areas in which proteases are used include pharmaceuticals, leather industry, in the production of protein hydrolysate, the film industry, and in waste processing companies.

The present study was planned to utilize MP as an inexpensive and rich medium for protease production, as a means to reduce its pollution potential as measured by the BOD.

MATERIALS AND METHODS

Cultures Maintenance

Four different protease-producing bacterial strains, *Bacillus megaterium* EMCC 1057, *Bacillus subtilis* EMCC 1020, *Pseudomonas fluorescens* EMCC 1221, and *Serratia marcescens* EMCC 1247, were used in this study. These cultures were obtained from the Egyptian Microbial Culture Collection (EMCC) at Cairo Microbiological Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Strains were propagated and maintained using tryptone soy broth (TSB).

Fermentation Media

Fresh MP was obtained from the Misr Milk and Food Co., Cairo, Egypt. Permeate was boiled for 30 min, separated, then centrifuged at about 6000*g* for 20 min, to remove all whey protein residue. The artificial media used were TSB, modified Luria broth (MLB), recommended by Zamost et al. (*8*), and the medium (TY) recommended by Malik and Mathur (*9*). The pH was adjusted to 7.0. Aliquots of 100 mL of the media were dispensed in 250-mL Erlenmeyer flasks, and sterilized at 121°C for 15 min.

Inoculum and Fermentation

Flasks containing culture media were individually inoculated with 5% of a 24-h culture (approx 10^7 cells/mL), and incubated at 30°C in a shaker water bath, with agitation at 120 rpm.

Analytical Procedure

Samples of broth were aseptically withdrawn at time zero and every 6 h for 48 h, and immediately analyzed for cell-mass concentration (culture growth), protease activity, lactose content, and BOD.

Cell-Mass Concentration

The growth of the microorganisms was monitored optically at 580 nm, using a Shimadzu spectrophotometer (Model UV-150-02, Shimadzu, Kyoto, Japan). Cell-mass concentrations were quantified by measuring sample optical density, and comparing readings with the cell-mass concentration prepared from a standard curve for each strain.

Protease Assay

The method reported by Quiros et al. *(10)* was used. A 120-μL portion of the sample was incubated at 45°C in a tube containing 480 μL 2% (w/v) azocasein (Sigmas, St. Louis, MO) in 0.2 *M* Tris-glycine buffer, pH 9.0, with 2 m*M* $CaCl_2$. After 30 min of incubation, the reaction was stopped by adding 600 μL 10% trichloroacetic acid, and the mixture was centrifuged. The precipitate was removed, and the supernatant was added to a tube containing 200 μl 1.8 *M* NaOH, and absorbance at 420 nm was measured. One unit of protease activity was defined as the amount of enzyme causing an increase in absorbance at 420 nm within 0.1h (6 min).

Lactose Concentrations

The concentration of residual lactose was measured using the phenol-sulfuric acid method described by Dubois et al *(11)*.

BOD Determination

The dissolved oxygen was determined using Corning instrument (Check made 90; Corning, NY). BOD_5 was expressed as indicated in *Standard Methods for the Examination of Water and Wastewater (12)*. The results presented were expressed as the average value of three replicates.

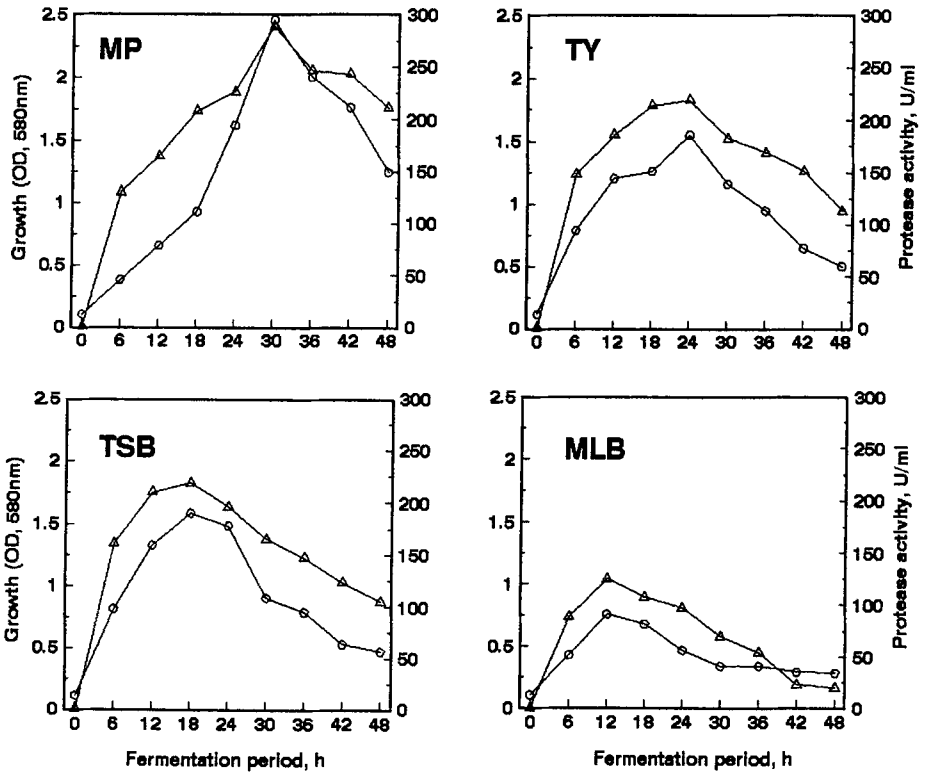

Fig. 1. Kinetics of growth (○) and protease production (Δ) by B. *subtilis* EMCC 1020 in different fermentation media; milk permeate (MP), medium of Malik and Mathur (TY), tryptone soy broth (TSB), and modified luria broth (MLB) at 30°C

RESULTS AND DISCUSSION

Figures 1–4 show kinetics of cell growth and protease production for *B. subtilis* EMCC 1020, *B. megaterium* EMCC 1057, *S. marcescens* EMCC 1247, and *P. fluorescens* EMCC 1221, respectively. *B. subtilis* protease reached its maximum levels after 12, 18, 24, and 30 h fermentation period in MLB, TSB, TY, and MP media, respectively. However, in MP medium, the maximum activity was greater (289 U/mL) than those in other fermentation media (Fig. 1). The same trend was also recorded for *B. megaterium* protease (Fig. 2). The protease of *S. marcescens* reached its maximum after 24 h in MLB, and after 30 h in all other media (Fig. 3). The maximum activity of *P. fluorescens* protease was reached after 24 h in MLB and TY, and after 30 h in MP and TSB media (Fig. 4). A correlation between the time of the maximum enzymatic production and the end of the cell exponential phase were observed for all organisms. Each of the organisms was able to grow in MP, and all produced considerable amounts of proteases. The protease activity of all organisms examined reached their maximum

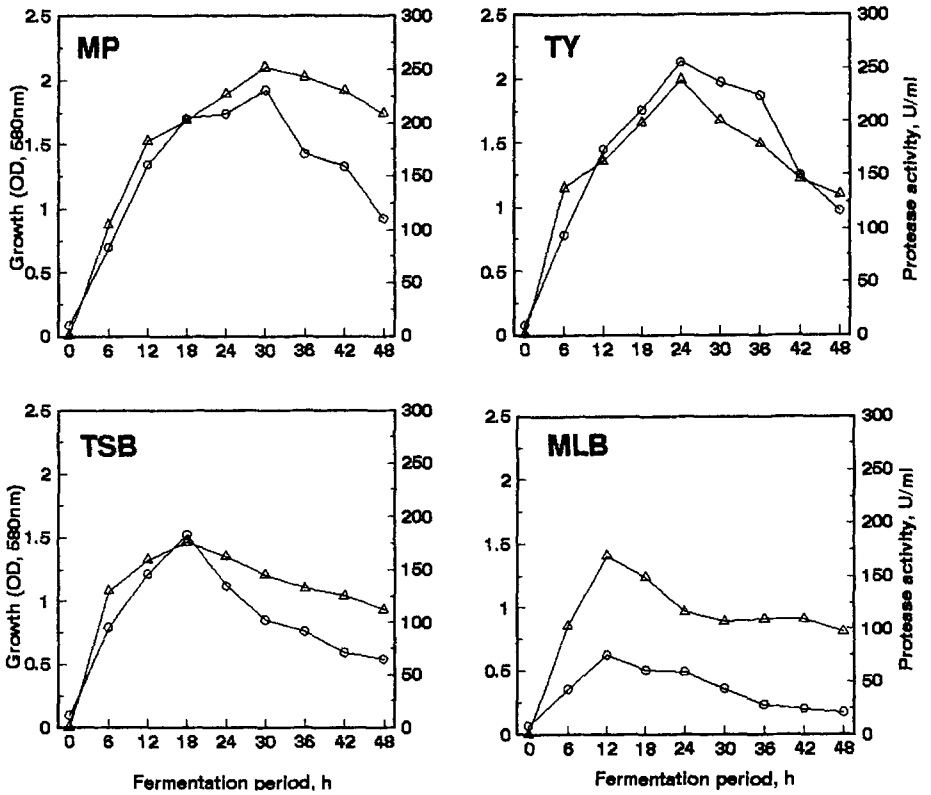

Fig. 2. Kinetics of growth (○) and protease production (Δ) by *B. megaterium* EMCC 1057 in different fermentation media: milk permeate (MP), medium of Malik and Mathur (TY), tryptone soy broth (TSB), and modified luria broth (MLB) at 30°C.

levels after 30 h fermentation period in MP. Activity then declined, so that, by 48 h, only 73.0, 82.9, 73.8, and 84.0% of the maximum levels remained for *B. subtilis, B. megaterium, S. marcescens,* and *P. flourescens* proteases, respectively.

All of the microorganisms reached their maximum growth and protese activity in MP after a relatively longer time (30 h) than those in other media. In MP, however, the growth and enzyme activity were greater than those in other fermentation media for all bacteria tested (Figs. 1–4). The results suggest that MP proved to be the most suitable medium for enzyme production by all organisms examined. These results are consistent with the findings of Quiros et al. *(10),* who found that whey presents the best medium for protease production by *S. marcescens* ATCC 25419, compared to the artificial media studied.

The efficiency of *B. subtilis, B. megaterium, S. marcescens,* and *P. fluorescens* for protease production in MP are presented in Table 1. The four bacterial strains produced considerable amounts of protease, reaching con-

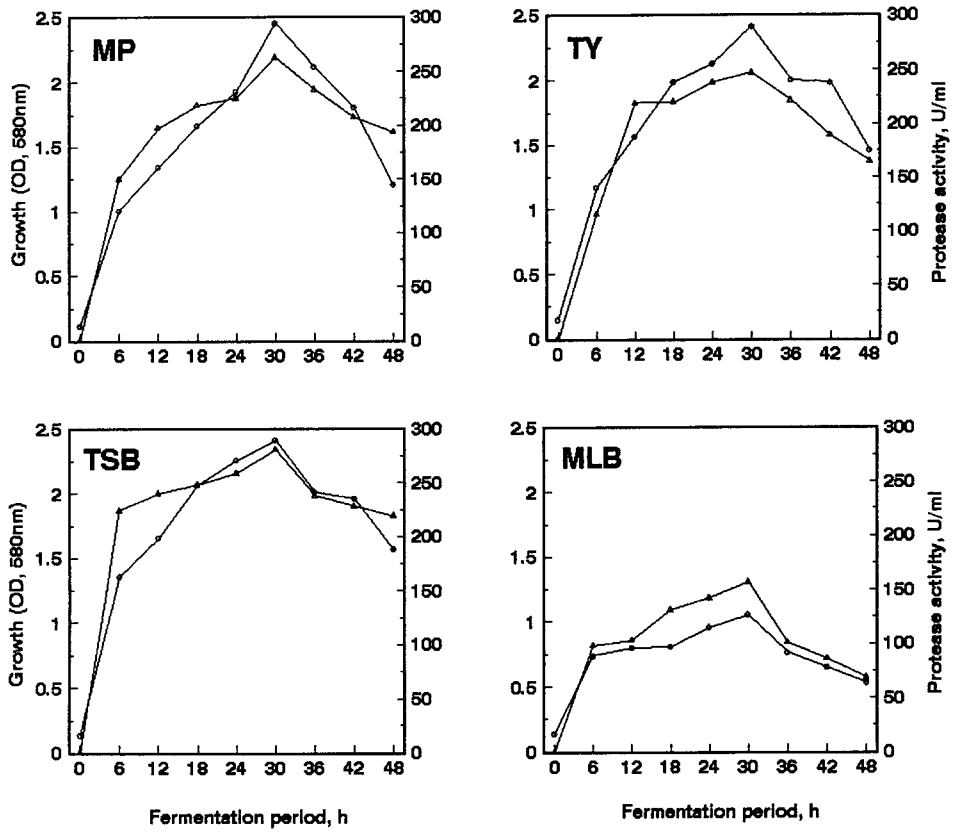

Fig. 3. Kinetics of growth (○) and protease production (Δ) by *S. marcescens* EMCC 1247 in different fermentation media: milk permeate (MP), medium of Malik and Mathur (TY), tryptone soy broth (TSB), and modified luria broth (MLB) at 30°C.

centrations of 289, 252, 263, and 212 U/mL, respectively, after 30 h of fermentation. Furthermore, the organisms were able to grow in MP, and produced considerable amount of biomass.

The *S. marcescens* strain showed higher protease/cell-mass yield (16.75 U/mg), followed by *B. subtilis, B. megaterium,* and *P. fluorescens,* in a decreasing order.

The pH values, lactose utilization, and BOD reduction by the protease-producing bacteria, grown in MP after a 30-h fermentation period, are shown in Table 2. The initial pH 7.0 reduced to 6.34, 5.48, 6.08, and 4.80 for *B. subtilis, B. megaterium, S. marcescens,* and *P. fluorescens,* respectively, at the end of the fermentation period. The protease-producing bacteria were able to utilize lactose in MP with values ranging from 37.62 to 54.97%. The highest value was recorded for *P. fluorescens* and the lowest was recorded for *B. megaterium.*

Table 1
The Efficiency of Protease Producing Bacteria for Protease Production in Milk Permeate

Strains	Biomass mg/mL	Protease production U/mL	Protease yield U/mg
B. subtilis EMCC 1020	18.25	289	15.84
B. megaterium EMCC 1045	17.05	252	14.78
S. marcescens EMCC 1247	15.70	263	16.75
P. fluorescens EMCC 1221	14.58	212	14.54

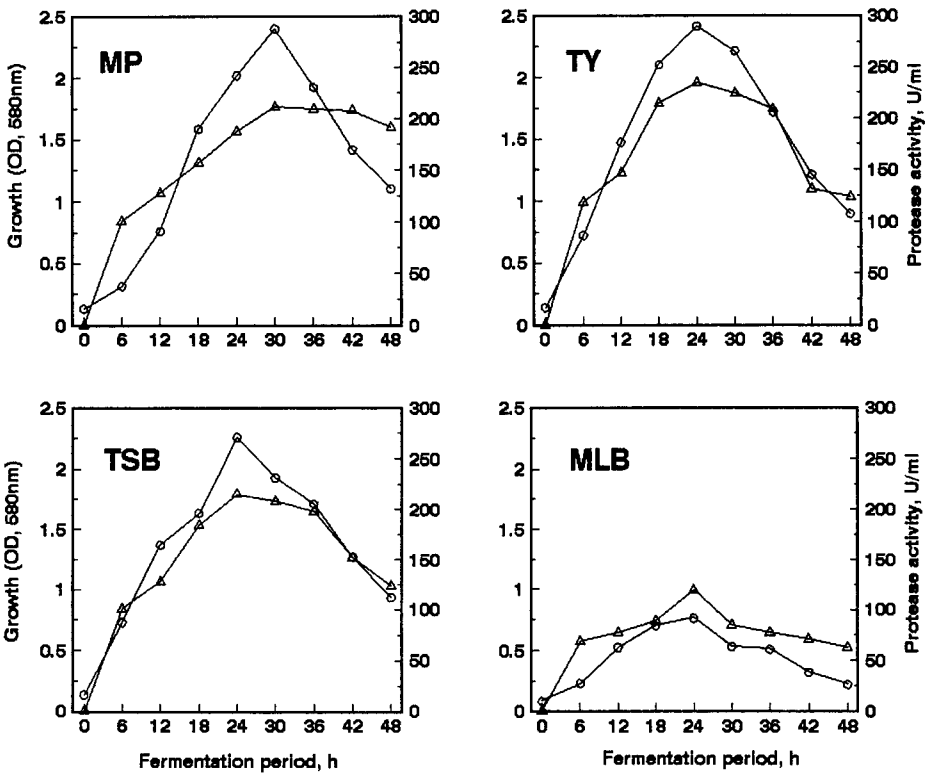

Fig. 4. Kinetics of growth (○) and protease production (Δ) by *P. fluorescens* EMCC 1221 in different fermentation media: milk permeate (MP), medium of Malik and Mathur (TY), tryptone soy broth (TSB), and modified luria broth (MLB) at 30°C.

Table 2
pH Values, Lactose Utilization, and BOD Reduction by Protease-Producing Bacteria in Milk Permeate[a]

Strains	final pH	Lactose utilized (%)	BOD reduction (%)
B. subtilis EMCC 1020	8.34	41.86	55.29
B. megaterium EMCC 1045	7.48	37.62	50.59
S. marcescens EMCC 1247	8.08	38.06	51.76
P. fluorescens EMCC 1221	6.80	54.97	63.65

[a]Initial milk permeate, pH = 7.0.

The organisms also reduced the BOD of MP. The results shown in Table 2 indicate that the BOD reduction increased as the lactose utilization increased. For all strains, the reduction of BOD varied from 50.59 to 63.65%. *P. flourescens* EMCC 1221 showed the highest BOD reduction.

Finally, the results reveal that the biomass produced, together with its intrinsic value, is a good source of proteases, which are of wide industrial and commercial interest. Since there is an inverse relationship between the amount of lactose utilized and the observed BOD value, the higher the amount of lactose utilized, the greater the reduction in the BOD value. The utilization of MP in this manner would be of great value in reducing environmental pollution.

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