Continuous production of a complex microbial consortium isolated from "Raclette" cheese surface using immobilized cell technology

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Introduction

Microorganisms play a central role in cheese manufacture from acidification of milk to development of complex organoleptic properties during the ripening period. The microflora associated with smear ripened cheese is very diverse and includes bacteria and yeasts belonging to different genera and species and sometimes moulds according to the cheese type. This microflora is traditionally propagated using the "old-young smearing method" which consists in transferring smear from old or ripened cheeses to young cheeses. However, a high risk of cheese contamination by pathogen or spoilage microorganisms is inherent to this procedure. On the other hand, contrary to defined food cultures that generally contain a low number of bacterial strains, large scale production of undefined cheese surface microbial consortia remains a challenging task from an industrial perspective. The aim of the present study was therefore to test the potential of immobilized cell technology and continuous cultures for the production of complex microflora, using as model the surface flora of Raclette cheese.

Materials and Methods

Cheese microflora

The cheese surface culture used in this study, originated from a smear-ripened Raclette cheese produced at an industrial Swiss dairy (Fromalp AG, Zollikofen, Bern). Samples of the complex surface microbiota were obtained by scraping off the smear with a scalpel and subsequently suspending in a solution of 3.3 % (w/v) NaCl and 15 % (v/v) glycerol using a stomacher (IUL, Spain). This complex culture is further referred to as FK.

Microflora immobilization

The surface culture was immobilized in polysaccharide gel beads, consisting of 2.5 % (w/v) gellan gum, 0.25 % (w/v) xanthan gum and 0.2 % (w/v) sodium citrate, using a double-phase dispersion process (Cinquin et al., 2004). The gel solution was inoculated with a 2-ml aliquot of the culture FK (inoculation rate of 0.66 % (v/v)). Beads with a diameter between 1-2 mm were collected by wet sieving, and washed with 0.1 M $CaCl_2$ solution.

Continuous fermentation with immobilized culture

Continuous fermentation was carried out in 1-L bioreactor (Multifors, Infors HT, Switzerland) with a total working volume of 700 ml, inoculated with 60 ml gel beads. The reactor vessel was continuously supplied with whey-based medium supplemented with casein peptone, yeast extract, sodium chloride and calcium chloride. The feed peristaltic pump was calibrated and flow rate set to achieve a dilution rate of 0.15 h^{-1}. The fermented medium was continuously withdrawn from the vessel using another peristaltic pump and the fermentation volume was controlled by positioning the outflow tube. To avoid a loss a gel beads from the reactor vessel, the harvest pipe was placed into a meshed basket attached to the impeller shaft.

Addition of acid (5 M HCl) and base (5 M NaOH) was used to control pH at 6.5, corresponding to a typical surface pH of ripened cheese. The foam level was controlled by addition of antifoam agent (10 % Antifoam A, Fluka Chemie AG, Switzerland). The fermentation was conducted at 25°C and with an agitation rate of 120 rpm. Aeration was done with sterile air continuously sparged in the medium at a flow rate of 1 liter per minute. The reactor was run in batch mode for the first 36 h to colonize gel beads. Thereafter, fermentation was run in continuous mode for 3 weeks.

Analysis of microbial population in the effluent

a. Culture dependent method

For the culture dependent analysis, samples of fermented medium were serially diluted in peptone water (Oxoid AG, Buchs, Switzerland), then plated in duplicate on seven different selective and non-selective agar media and incubated under conditions suitable for each medium (table 1). After incubation the number of colony forming unit was determined and expressed as CFU/ml or CFU per gram of gel beads.

Name	Description	Specificity	Incubation conditions
SC	Standard methods agar[1] + 1 % (w/v) casein peptone[2]	Aerobic mesophilic count	Aerobic, 3 days at 30°C, then 7 days at 25°C with light exposure
BP	Baird Parker agar[3] + egg yolk tellurite supplment[3]	Staphylococci	Aerobic, 6 days at 37°C
MSA	Mannitol salts agar[1]	Staphylococci	Aerobic, 6 days at 37°C
PY	Phytone yeast extract agar[1]	Yeasts/ moulds	Aerobic, 6 days at 30°C
MRS	De Man Rogosa Sharpe agar[3] + Tween 80[3]	Lactic acid bacteria	Anaerobic, 6 days at 37°C
PALCAM	Palcam agar base[1] + Palcam selective supplement[1]	*Listeria* sp.	Aerobic, 3 days at 37°C

Suppliers: [1]Difco™ Labatories, France; [2]Merck, Germany; [3]Biolife; [4]Oxoid AG, Buchs, Switzerland

Table 1: Media and incubation conditions

b. Culture independent method: Temporal Temperature Gel Electrophoresis (TTGE)

Bacterial diversity in FK, fermented medium and on the different agar media was analysed using TTGE. This technique consisted first in PCR amplification of a 200 base pairs fragment from V3 variable region of the 16S rDNA. The PCR products were then separated according to their GC content, using TTGE, and visualized by staining gel with ethidium bromide and UV exposure. Bacterial populations belonging to the same genus or species produce a specific bands pattern after gel electrophoresis.

Results & Discussion

Biomass production in the continuous immobilized cell bioreactor

The schematic diagram of the fermentation process is showed on figure 1. Biomass production in the reactor effluent originates from the continuous growth and release of cells from gel beads surface and growth of free cells in the bulk liquid (Lacroix et al., 2004). The high cell density typically reached in gel beads (ca. 10^{11} CFU/g) provides an efficient and stable inoculation of the circulating medium, as shown with complex gut fermentation (Cinquin et al., 2006).

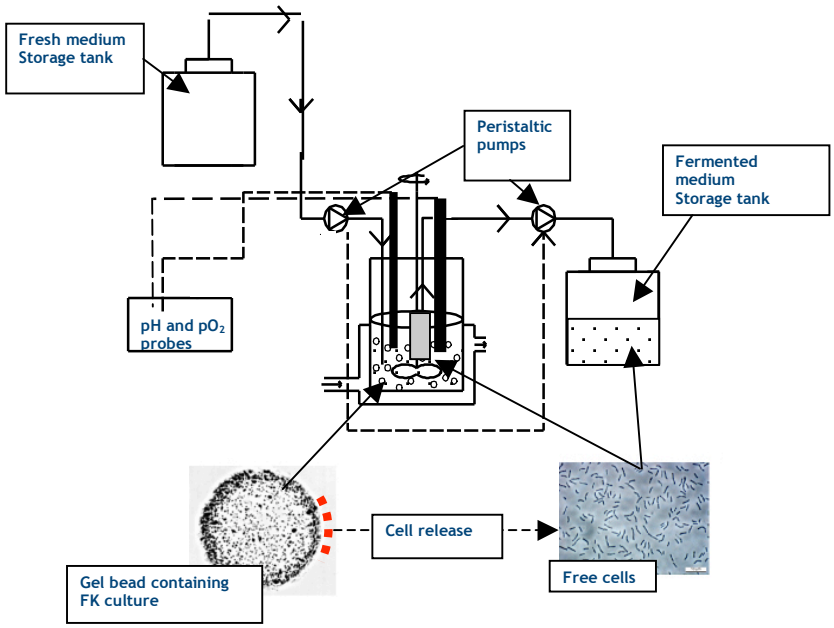

Figure 1: Schematic diagram of continuous fermentation using immobilized cell for production of cheese surface microflora.

Analysis of microbial populations

Cell counts of mesophilic aerobic bacteria and Staphylococci on MSA in reactor effluent were stable and high and slightly lower than in initial FK culture (Figure 2a).

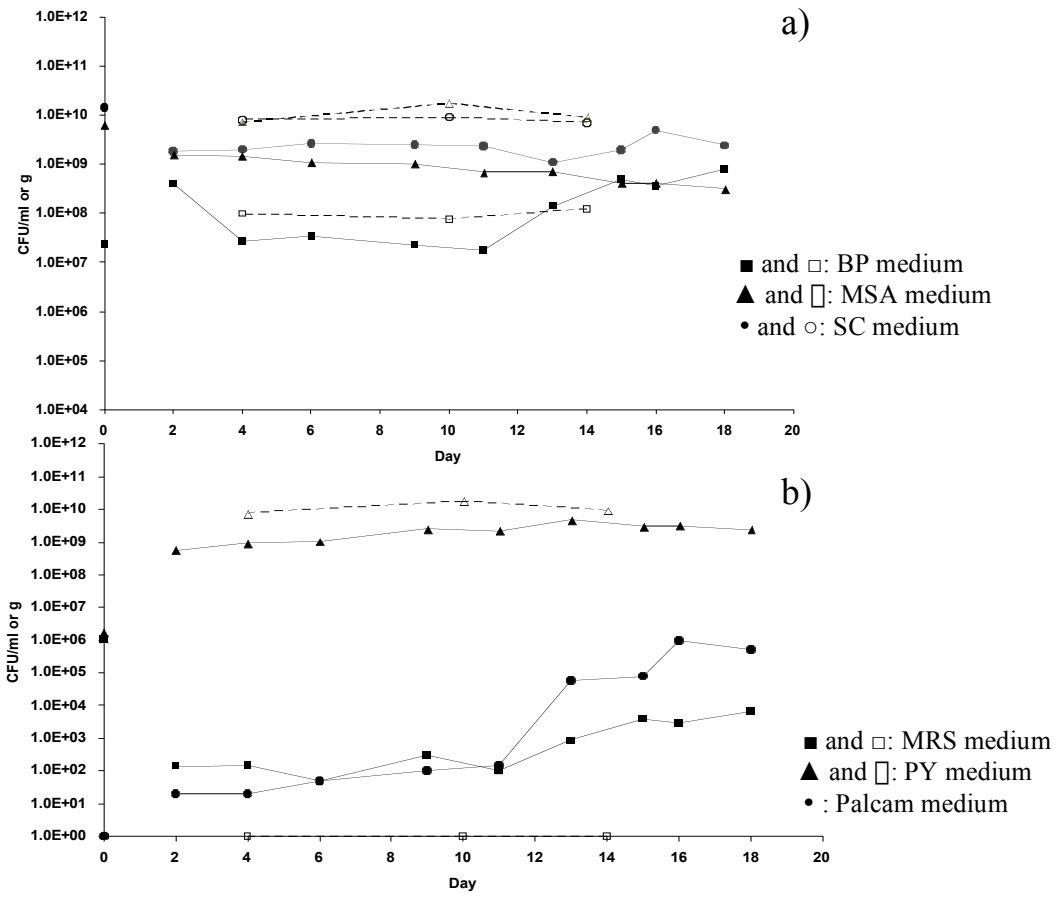

Figure 2: Populations in initial FK culture (separate points at day 0), in fermented medium (continuous line, closed symbols) and gel beads (dashed line and open symbols) determined using different media (indicated on the right side of each figure).

Staphylococci counts in effluent samples (BP medium) were similar to that in FK during day 4 to 11 of continuous culture and increased to 1.10^9 CFU/ml after 15 day to remain constant afterwards (Figure 2a). Lactic acid bacteria cell counts did not changed with fermentation time and remained about 3 log higher than in FK (Figure 2b). Immobilized populations of mesophilic aerobic bacteria, lactic acid bacteria and Staphylococci (BP and MSA media) remained constant and high during fermentation at 1.10^{10} CFU per gram of gel beads (Figures 2a and 2b). Yeast population was lower in effluent compared to FK but increased with fermentation time to reach 1.10^4 CFU/ml after 18 days (Figure 2b). Surprisingly, yeasts were not detected in gel beads (Figure 2b). Cell counts on Palcam medium increased with time. Palcam was used to detect *Listeria* but some *Enterococcus* sp. could also grow on this selective medium, as confirmed using PCR.

The observed differences for population counts in effluent and initial FK culture can be explained by the fixed operating conditions in the reactor, such as aeration rate which is a limiting factor for development of yeasts. Furthermore, large changes in microbial population ratio of cheese surface microflora also occur during cheese ripening period.

Analysis of the microflora in continuously fermented medium using a powerful molecular method, TTGE, indicated that microbial diversity of the original smear microflora was kept in effluent during the continuous fermentation (Figure 3).

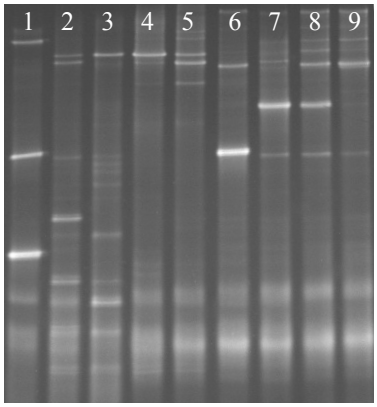

Figure 3: Low GC-TTGE with samples from initial FK culture (lane 2), FK plated on SC (lane 3), MSA (lane 4), BP (lane 5) and MRS (lane 6), and fermented samples after 1, 2 and weeks (lane 7, 8 and 9, respectively) of continuous fermentation with immobilized cells. Lane 1: marker. The figure is representative of several gels.

Conclusion

Our study indicated that immobilized cell technology and continuous fermentation can be used to produce complex undefined cheese surface microflora without detectable change in the biodiversity. Further experiments will be done to evaluate the potential of this technology for the controlled one step production of complex cheese surface cultures and to test the application of such cultures for cheese manufacturing and ripening.

References

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