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FLOW CYTOMETRIC ANALYSIS OF MICROBIAL CONTAMINATION IN FOOD INDUSTRY TECHNOLOGICAL LINES – INITIAL STUDY

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ABSTRACT

Background. Flow cytometry constitutes an alternative for traditional methods of microorganisms identification and analysis, including methods requiring cultivation step. It enables the detection of pathogens and other microorganisms contaminants without the need to culture microbial cells meaning that the sample (water, waste or food e.g. milk, wine, beer) may be analysed directly. This leads to a significant reduction of time required for analysis allowing monitoring of production processes and immediate reaction in case of contamination or any disruption occurs. Apart from the analysis of raw materials or products on different stages of manufacturing process, the flow cytometry seems to constitute an ideal tool for the assessment of microbial contamination on the surface of technological lines.
Material and methods. In the present work samples comprising smears from 3 different surfaces of technological lines from fruit and vegetable processing company from Greater Poland were analysed directly with flow cytometer. The measured parameters were forward and side scatter of laser light signals allowing the estimation of microbial cell contents in each sample.
Results. Flow cytometric analysis of the surface of food industry production lines enable the preliminary evaluation of microbial contamination within few minutes from the moment of sample arrival without the need of sample pretreatment.
Conclusions. The presented method of flow cytometric initial evaluation of microbial state of food industry technological lines demonstrated its potential for developing a robust, routine method for the rapid and labor-saving detection of microbial contamination in food industry.

Key words: flow cytometry, microorganisms detection, microbial contamination, cell sorting

INTRODUCTION

Flow cytometry allows multiparametric analysis of cell morphological features, interactions between cellular molecules and qualitative and quantitative evaluation of environmental microbial composition directly in analysed material and without the need to proceed with cultivation step. Flow cytometer measures suspended cells and particles flowing through the flow cell (flow chamber). The cells from analysed suspension, converted into fluid stream, are being scanned by intercepting laser beam as they pass through the observation point (interrogation point). The measured parameters are laser light scatter and emission of light of fluorochromes (fluorescence) excited by laser beam [Shapiro 2003]. Flow cytometer enables the simultaneous measurement of fluorescence emission intensity (coming from excited fluorochromes tagging directly specific cell structure or conjugated with antibodies or lectins) and laser light scattered on suspended cells.

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Flow cytometry calls the cell or particle, intercepted by laser beam, an event and modern flow cytometers are able to analyze up to 100 000 events per second measuring several parameters (fluorescence and light scatter) for each event. The main advantage of flow cytometry is rapid, objective and fully automated measurement of single cell in the whole population and detection of trace subpopulations. Thus the application of flow cytometer constitute a powerful tool to rapidly enumerate microorganisms leading to simultaneous discrimination between viable, metabolically active and dead cells, which is of great importance in food processing. Compared to other analytical tools, where a single value for each parameter is obtained for the whole population, flow cytometry provides data for every particle detected [Comas-Riu and Rius 2009].

Flow cytometric measurement of microorganisms concerns microbial cell identification, cellular molecules interaction, multiparametric analysis of complex populations, study of environment variations influence on microbial cells (the impact of antibiotics, bacteriocins and disinfecting agents) and evaluation of transformation process using reporter fluorescent proteins as markers. Common cell parameters employed in flow cytometric measurements of microorganisms constitute cell membrane integrity allowing for live/dead cell discrimination, differences in DNA and RNA structure, metabolic activity in the form of respiration in aerobic bacteria which can be detected using an indicator of oxidative metabolism such as a cyanoditolyl tetrazolium chloride (CTC), intracellular pH (potentially providing information about viability) with the use of some fluorescent probes such as carboxyflouorescein diacetate (CFDA) [Comas-Riu and Rius 2009]. Additionally flow cytometry offers an opportunity to sort analysed cells, which is an invaluable asset for microbiology studies. It can be used to confirm that a particular population is what you think it is, and for the isolation of target populations for further characterization [Veal et al. 2000].

Spoilage and pathogen microorganisms contamination of food products is a major concern of industries involved in food transformation. The presence of microbial cells like bacteria, yeasts and moulds in final products can severely affect their quality, independently of their concentration. Detection of food contamination by microorganisms should be rapid and informative thus the search for an ideal method focuses on finding the procedure simple and labor-saving.

The aim of the present study was an evaluation of preliminary method enabling the rapid assessment of the state of microbial contamination in food industry with the particular emphasis on food processing lines being a source of food spoilage and responsible for production process interferences including the necessity to withdraw a final product.

MATERIAL AND METHODS

Preparation of surface samples. The analysed samples comprised smears from 3 different surfaces (plastic belt and stainless steel conveyors) of technological lines from fruit and vegetable processing company from Greater Poland. Smears covered the area of 100 cm^2 (10 × 10 cm). Swabs were placed in 3 ml of 1% PBS solution and transferred to flow cytometric laboratory of Department of Biotechnology and Food Microbiology for analysis.

Organisms and growth conditions. Additional analysis of one of the samples (sample 3) involved mixing with yeast cells and subsequent flow cytometric measurement. Another analysis involved sample filtration with the use of 0.45 µm pore-size syringe filter. Lyophilized cells of *Saccharomyces cerevisiae* strain Red Star Ethanol Red (Lesaffre, France) were added in a concentration of 0.5 $g \cdot l^{-1}$, corresponding to 2.5×10^6 $cfu \cdot ml^{-1}$ after inoculation, to the YPD medium in a 500-ml Erlenmeyer flask and incubated at 30°C overnight with orbital shaking at 125 RPM. After incubation 2 µl (approx. 2×10^5 cells) of yeast culture were added to sample 3 and mixed by vortexing.

Flow cytometry protocols. Samples analysis were performed directly (no pretreatment stage employed) with flow cytometer (cell sorter) BD FACS Aria™III (Becton Dickinson), equipped with 4 lasers (375 nm, 405 nm, 488 nm and 633 nm), 11 fluorescence detectors, forward scatter (FSC) and side scatter (SSC) detectors. Primary sample line was fitted in initial 35 µm-pore-size filter, preventing flow arrest as samples may include food-borne particles capable to block the light of sample line or nozzle. The instrument setup (optical alignment), stability and performance test were made using CST system (Cytometer Setup and Tracking)

from Becton Dickinson company. In analysis non-fluorescent parameters (forward and side scatter) were employed. FACSFlow solution (Becton Dickinson) was used as sheath fluid. The configuration of flow cytometer was as follows: 70 µm nozzle and 70 psi sheath fluid pressure. The flow cytometry analyses were performed by using logarithmic gains and specific detectors settings. The threshold was set on the FSC signal. Data were acquired in a five-decade logarithmic scale as area signals (FSC-A and SSC-A) and analysed with FACS DIVA software (Becton Dickinson). The populations were defined by gating in the dot plots of FSC and SSC. Each sample was analysed in tetraplicates. Flow cytometric counts of events expected as bacterial cells were obtained by normalizing the numbers of events occurring in region on dot plots (gate P1) that defined the estimated bacterial cell population to the volume of sample analysed. Number of events in gate P1 for each sample constitute approximately 60% of all collected events. In order to assess the cellular content (bacteria and yeast cells) in analysed samples events were gated and sorted with the use of BD FACS Aria™III cell sorter. Sort regions were defined on bivariate dot plot (FSC-A versus SSC-A) that delineated two distinct populations. Cell sorting preceded doublets discrimination procedure with the use of height versus width scatter signals measurement, in order to discriminate single events from conglomerates. Doublets discrimination procedure comprised two step gating of events to encompass low SSC-W population on SSC-H vs. SSC-W dot plot and low FSC-W population on FSC-H vs. FSC-W dot plot. Sorting involved singlets of P1 (singlets 1) and P3 gates (singlets 2). Sorted populations were then examined for the microbial cell content with the use of crystal violet staining and microscopic observations.

RESULTS AND DISCUSSION

For three samples yielded from three different surfaces of food industry processing lines estimated numbers of microorganisms were measured using forward and side scatter signals gained as signal area (Fig. 1). Events from gate P1 (anticipated bacterial cells) were counted by normalizing the number of events to the volume of sample analysed. The number of events counted was then converted to counts per cm^2 of the analysed surface. Means of estimated bacterial cell counts × cm^{-2} of the surface for three different samples (sample 1, 2 and 3) demonstrates Figure 2. The comparison of counted events from P1 gate normalized to the volume of sample analysed allows the evaluation of bacterial cell number within few minutes from the moment of sample arrival. The results of P1 gate event counts for samples 1, 2 and 3 revealed statistically significant differences in estimated bacterial cell numbers between samples. Sorting experiments performed for analysed samples aimed at discrimination between microbial cells and the background containing organic and inorganic particles. Populations selected for sorting were defined on bivariate dot plot (FSC-A versus SSC-A) that delineated two distinct populations. First population (gate P1) assumed to comprise bacteria encompassed approximately P1 gate from the number of events measurement. The second defined population (gate P3) evaluated as larger and more complex cells (yeast cells) demonstrated higher signals for FSC and SSC. The Figure 3 shows gated populations of sample 1, analysed and prepared for sorting – doublets discrimination procedure involved (gates P2 and singlets 1 as well as P4 and singlets 2 are hierarchical gates derived from P1 and P3 gates respectively). Microscopic pictures of sorted events stained with crystal violet demonstrates Figure 4. Microscopic observations revealed microbial cells: bacteria – events gated as singlets from P1 gate (singlets 1; Fig. 4 a, 4 b) and yeast – events gated as singlets from P3 gate (singlets 2; Fig. 4 c, 4 d), which constitute an evidence that calculated events comprise cells of microorganisms (mainly bacteria).

An additional analysis of one of the samples (sample 3) involving mixing with yeast *Saccharomyces cerevisiae* cell culture and filtration of sample 3 with the use of 0.45 µm pore-size syringe filter were employed (Fig. 5). Both analysis aimed at determining that the population of events representing anticipated bacterial cells comprise bacterial content of analysed samples, as showed prior analysis involving the evaluation of mutual position of yeast and bacteria populations on FSC vs. SSC dot plot with the identical detectors settings. Sample filtration resulted in significant decrease of collected events, however counts for yeast cells diminished at higher rate than bacterial cells.

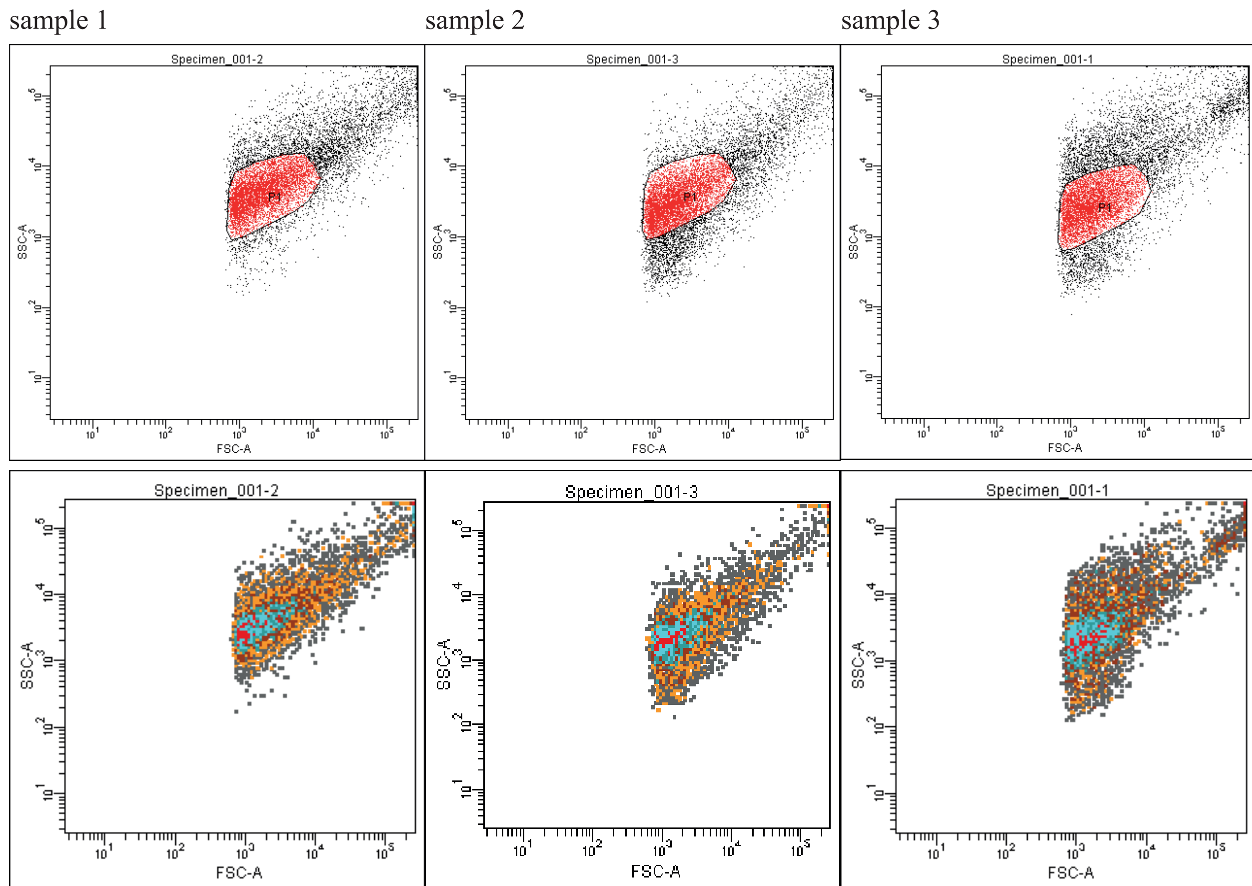


Fig. 1. Flow cytometric analysis of estimated microbial cell number in samples from 3 different surfaces of technological lines in fruit-vegetable processing company (samples 1, 2 and 3). Sample analysis were performed directly (no pretreatment stage employed) with flow cytometer (cell sorter) BD FACS Aria™III (Becton Dickinson). In analysis forward scatter (FSC) and side scatter (SSC) measurements were applied, data were collected as area signals (FSC-A and SSC-A) and analysed with FACS DIVA software (Becton Dickinson). FSC and SSC parameters are designated to cells size and complexity. The estimation of microbial cell count for each sample was conducted by normalizing the numbers of events occurring in region on dot plots (gate P1) that defined the estimated bacterial cell populations to the volume of sample analysed

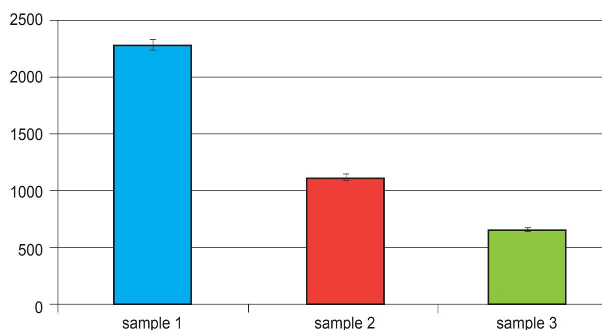


Fig. 2. Means of estimated bacterial cell counts per cm^2 of the analysed surface. Samples were collected from three different surfaces of fruit and vegetable processing company production lines (sample 1, 2 and 3). Each sample was analysed in tetraplicates. The comparison of counted events from P1 gate normalized to the volume of sample analysed allows the evaluation of estimated bacterial cell number within few minutes

sample 1

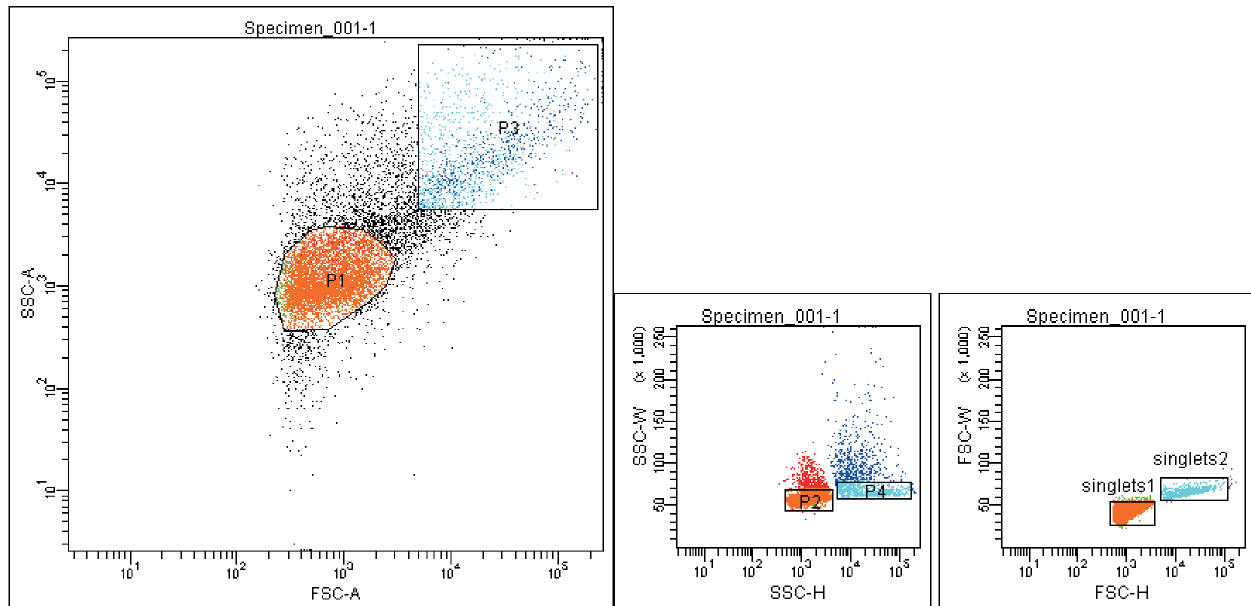


Fig. 3. Sorting of the analysed events from one of the sample (sample 1) in order to assess the cellular content (bacteria and yeast) of samples. The first sorted population expected to comprise bacteria (gate P1) encompassed approximately P1 gate from the number of events measurement (FSC-A vs. SSC-A dot plot). The second population evaluated as larger and more complex cells (higher signals for FSC and SSC) was gated (gate P3). In both cases cell sorting preceded doublets discrimination procedure with the use of height versus width scatter signals measurement, in order to discriminate single events from conglomerates (SSC-H vs. SSC-W and FSC-H vs. FSC-W dot plots). Gates P2 and singlets 1 as well as P4 and singlets 2 are hierarchical gates derived from P1 and P3 gates respectively. Sorting involved singlets of P1 (singlets 1) and P3 gates (singlets 2). Sorted populations were then examined for the microbial cell content with the use of crystal violet staining and microscopic observation

This is an anticipated consequence of yeast cells being larger and more complex compared to bacterial cells.

The present work is an innovative approach to rapid and labor-saving detection of microorganisms directly on the surfaces of production lines. This preliminary analysis of microbial contamination of technological lines in food industry, which was performed with the use of a flow cytometer, demonstrated a considerable potential for the rapid assessment of the microbiological state of surfaces involving food processing. The method presented in this study does not require any time-consuming pre-treatments. The analysis enabled only the estimation of the number of microbial cells but the time needed for analysis to take place was much shorter than in the case of traditional microbiological methods, including microscopic observation. Techniques based on flow cytometric principles are routinely applied in the dairy industry for measuring the total bacterial count within a few minutes [Gunasekera et al. 2000, 2003]. Even with the use of fluorescent staining procedures the results of flow cytometric approach can be obtained in 20-30 min [Assuncao et al. 2007]. An example of the simplicity of detection methods is the cytometric differentiation between Gram positive and negative bacteria. A combination of two DNA binding fluorescent dyes is employed for this purpose: SYTO 13, which penetrates easily cellular membranes and HI (hexidium iodide), which is blocked by lipopolysaccharide layer of Gram negative bacteria and is, thus, only permeable to Gram-positive bacteria and Gram-negative bacteria with a destabilized lipopolysaccharide layer [Mason et al. 1998]. A similar technique involves the use of Oregon Green-conjugated wheat germ agglutinin (WGA) with HI.

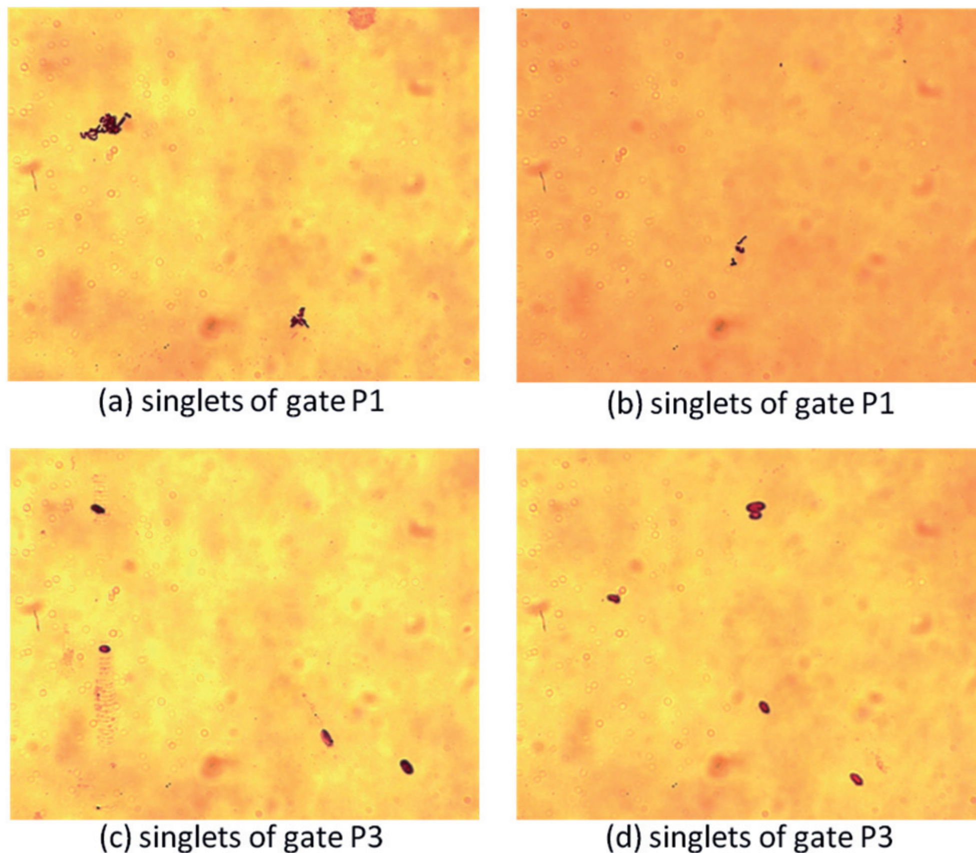
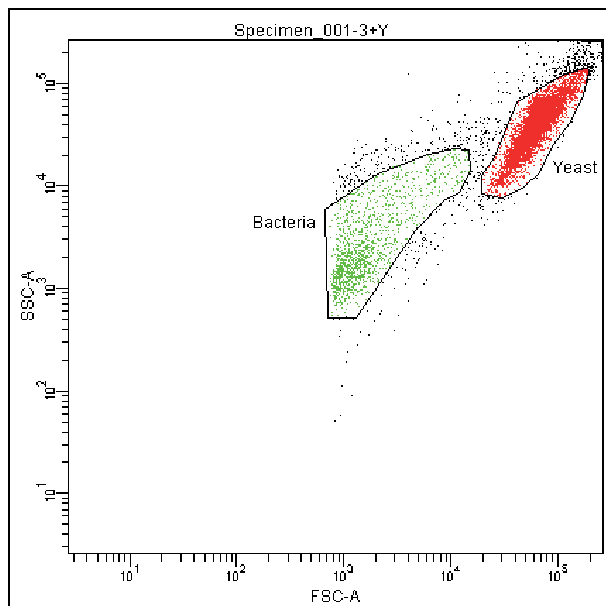


Fig. 4. Microscopic pictures of sorted cells from sample 1 stained with crystal violet. Sorting aimed at assessing the cellular content (bacteria and yeast) within analysed populations. Sorting involved singlets of P1 (expected bacterial cells) and P3 gates (expected larger and more complex cells). Microscopic observations revealed microbial cells (bacteria – events gated as singlets from P1 gate and yeast – events gated as singlets from P3 gate), which constitute an evidence that calculated events comprise cells of microorganisms (mainly bacteria)

WGA binds to *N*-acetylglucosamine in the peptidoglycan layer of the cell wall of Gram-positive bacteria whereas HI binds to the DNA of all bacteria after permeabilization by EDTA and incubation at 50°C for 15 min [Holm and Jespersen 2003]. Using WGA instead of SYTO 13 and treating the cells with EDTA allows the latter technique to be used directly in milk samples without precultivation of the sample [Holm and Jespersen 2003, Holm et al. 2004]. Another low-complex method that can be employed for microbial cell characterization is the mentioned use of redox potential indicators such as cyanoditolyl tetrazolium chloride (CTC). In combination with dyes enabling the live/dead cell discrimination this technique constitutes a rapid and, thus, powerful tool in the estimation of microbial cell content and their metabolic potential in an analysed sample. Further stage of the designed experiment engages the use of fluorescent cell staining in order to discriminate between cells and the background, as well as to assess the viability and metabolic activity of microbial cells.

Flow cytometry application in food industry involves characterization of microbial populations detecting live, dead or metabolically active cells. Recent studies have shown that the viable or active but non-cultivable forms of microbes are becoming

sample 3a



sample 3b

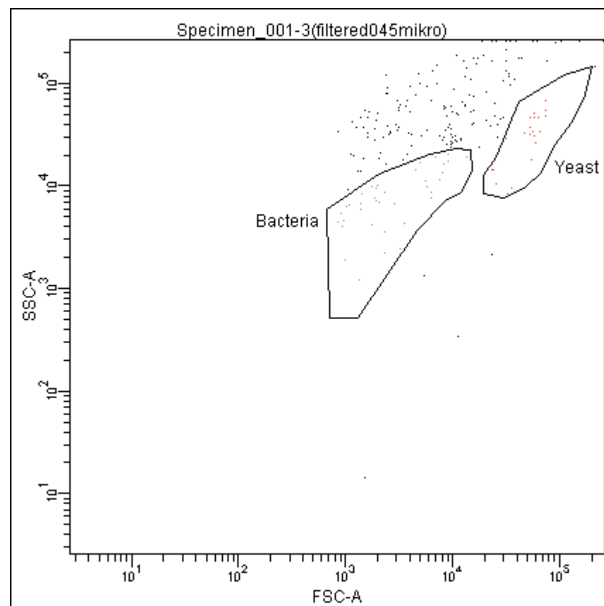


Fig. 5. An additional flow cytometric analysis of sample 3, which involved mixing with yeast *Saccharomyces cerevisiae* cells culture (sample 3a) and sample filtration with the use of 0.45 μm pore-size syringe filter (sample 3b). Both analysis aimed at determining that the population of events representing anticipated bacterial cells constitute bacterial content of analysed samples, as showed prior analysis involving the evaluation of mutual position of yeast and bacteria populations on FSC vs. SSC dot plot with the identical detectors settings

of increasing concern in environmental microbiology including technological surfaces of food industry. A formation of active but non-cultivable state, where the cell remains capable of undergoing metabolic activity but cannot be detected by cultivable methods, constitute a feature of non-sporulating microorganisms [Barer et al. 1993, Rozak and Colwell 1987, McDougald et al. 1998]. This phenomenon was largely demonstrated based on the discrepancies observed between culturing methods and non-culture-based staining techniques [Baudart et al. 2002, McNamara et al. 2002, Rice et al. 2000]. Production lines are believed to constitute a rich source of microbes, which remains largely undetected using cultivable methods. Flow cytometry being a non-culture dependent technique, should be able to differentially measure the active but non-cultivable bacteria [Sachidanandham et al. 2005].

Particular significance has an evaluation of microbial state on the surface of food industry technological lines. These elements comprise the source of contamination on different stages of food processing thus the compilation of rapid and labor-saving methods enabling the analysis of cleaning and disinfecting procedures efficiency, is essential from the economic point of view and might interest food production companies. The rapid analysis of the surface of food technological lines leading to monitoring of the production process allows selection of optimal cleaning and disinfecting agents and procedures [Juzwa 2011]. Methods for rapid detection of microbial contamination enable primary preventing of production failures as a consequence of any occurring contamination or disruption.

CONCLUSIONS

Food industry production lines can be the source of bacterial contamination on different stages of the manufacturing process, meaning that the compilation of time and labor-saving methods, allowing the analysis of cleaning and disinfecting procedures efficiency, is essential also from the economic point of

view. The significant advantage of flow cytometric analysis comprise the opportunity of omitting the cultivation step and the multi-parametric measurement of various features of microbial cells including species or strain identification. The use of flow cytometer in food industry enables monitoring of different stages of production process leading to reduction of process interferences associated with the production arrest or the necessity to withdraw a final product.

Method of flow cytometric initial evaluation of microbial state of food industry technological lines suggested in this study represents an innovation. Its application for the time being is limited as costs of instrument purchase are still considerable. However the accessibility of flow cytometers increases and their prices diminishes making flow cytometric detection of microorganisms an alternative in the near future. This work constitute a first step in developing a robust, routine flow cytometric method for the rapid detection of microbial contamination in food industry. Next step involves the flow cytometric detection of metabolic state and viability of microbial cells with the use of fluorescent staining.

SUMMARY

The possible applications of flow cytometry in food industry involve the microbiological analysis of food production processes including the state of technological lines. Determination of microbial state of the production lines surface is crucial for detailed evaluation of contaminants. Flow cytometry has become a valuable tool in food microbiology allowing the reduction of time required for analysis and monitoring food processing stages from raw material to final product release.

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CYTOMETRYCZNA ANALIZA ZANIECZYSZCZEŃ MIKROBIOLOGICZNYCH LINII TECHNOLOGICZNYCH W PRZEMYŚLE SPOŻYWCZYM – STUDIUM WSTĘPNE

STRESZCZENIE

Wprowadzenie. Cytometria przepływowa jest alternatywą dla klasycznych sposobów identyfikacji i analizy mikroorganizmów, w szczególności metod hodowlanych. Pozwala na wykrywanie patogenów oraz innych niepożądanych mikroorganizmów bez konieczności ich hodowli, czyli bezpośrednio w pobranym materiale (woda, odpady oraz żywność, np. mleko, wino, piwo). Prowadzi to do znaczącego skrócenia czasu trwania analizy, pozwalając na monitorowanie procesów produkcji i szybką reakcję w przypadku pojawienia się zakażeń lub jakichkolwiek zakłóceń. Poza analizą surowców oraz produktów na różnych etapach procesu ich wytwarzania, cytometria przepływowa jest idealnym narzędziem do oceny zanieczyszczeń mikrobiologicznych powierzchni linii technologicznych.
Materiał i metody. W prezentowanej pracy próbki – stanowiące wymazy z trzech powierzchni linii technologicznych zakładu przetwórstwa owocowo-warzywnego z terenu Wielkopolski – były analizowane bezpośrednio z zastosowaniem cytometru przepływowego. Określanym parametrem było rozproszenie światła lasera mierzone przez przedni oraz boczny detektor światła rozproszonego, co umożliwiło oszacowanie ilości komórek mikroorganizmów w pobranych próbkach.
Wyniki. Cytometryczna analiza powierzchni linii produkcyjnych przemysłu spożywczego pozwala na wstępne określenie zanieczyszczenia mikrobiologicznego w czasie kilku minut od momentu przywiezienia próbki, bez konieczności jakiejkolwiek obróbki.
Wnioski. Zaprezentowana analiza wstępnego określania stanu mikrobiologicznego linii technologicznych w przemyśle spożywczym z zastosowaniem cytometrii przepływowej wykazała możliwość opracowania solidnej, ułatwiającej wykorzystanie rutynowe, metody detekcji zanieczyszczeń mikrobiologicznych w przemyśle spożywczym.

Słowa kluczowe: cytometria przepływowa, detekcja mikroorganizmów, zanieczyszczenia mikrobiologiczne, sortowanie komórek

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