International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 5 No. 4 Apr. 2014, pp. 333-343

http://www.ijias.issr-journals.org/

Potential use of flow cytometry in microalgae-based biodiesel project development

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ABSTRACT: The decrease of petroleum reserves and the global increase in energy demand has resulted in the focus of research toward exploration of alternate fuels using biological renewable sources. Biodiesel is one of these renewable energy forms. Microalgae are considered one of the most promising sources for biodiesel production. However, the potential use of microalgae in this field still needs to be explored since only about twenty species has been studied from the approximately 30.000 known species. Thereby, screening work on new potentially lipid overproducer strains is actually an active field. An overview of potential applications of multi-parameter flow cytometry in development of a biodiesel production strategy using microalgae is detailed in this review. Rapid and simultaneous measurements of different physiological parameters indicating diversity in marine and freshwater microalgal communities, biomass quality such as biochemical composition and viability of individual cells, isolation of targeted cells and obtention of axenic cultures are applications of flow cytometry that enable monitoring and optimisation of production of lipids from microalgae.

KEYWORDS: microalgae, biodiesel, flow cytometry, fluorescence, screening.

1 INTRODUCTION

Development of biofuels using microalgae as a feedstock is increasingly studied worldwide. This is a consequence of an increase in global energy demand, a decline in global petroleum reserves as well as the increased global awareness towards the development of renewable energies respectful of the environment. Microalgae are a desirable source for biofuels production compared to other biofuel feedstocks in view of the following strengths: **(i)** microalgae have a fast growth rate, **(ii)** microalgae culture can use non fresh water (marine or waste water) and non-arable lands **(iii)** microalgal based biofuels do not interfere with food security concerns, in comparison with first generation biofuels, **(iv)** lipid metabolism can be easily modulated by modifying growth conditions allowing a significant increase in lipid content, and finally **(v)** intracellular lipid content can rise 80% of cell dry weight making microalgae an attractive source for biodiesel production [1], [2], [3]. Thereby, microalgae seem to be a promising renewable biofuel source capable of meeting the global demand for transportation fuels [1].

Studies on microalgae candidates for biodiesel projects have focused only on around twenty species [1, Tab. 1], which indicates a limited exploration of the wide algal biodiversity. This could be due to: **(i)** the ease of working with known strains whose taxonomy, culture requirements are well described and related documentation available, **(ii)** culture conditions (media, light intensity, CO_2 supply, response to stress, etc.) have been widely studied which means time and effort saving, **(iii)** metabolic profiles are well known in most of the cases, (**iv)** some species are considered models for genetic manipulations and engineering, **(v)** Some of these species have even been tested in pilot scale and used by industrials, which encourages researchers to work on, for improving productivity to the detriment of new species. However, it is essential to expand the

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spectrum of studied strains, in order to develop economically viable biofuel projects. This can lead to find new lipid-rich species, in addition to isolating indigenous homologues of known species. In consequence, various projects around the world have undertaken programs of high-throughput screening in order to isolate homologues of known species and to explore new ones [4], [5].

Table 1. Oil content of some microalgae [1]

Microalga	Oil content (% dry wt)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis suecica	15–23

Despite the significant progress in the field of microalgae-based biodiesel, several technical barriers need to be overcome for a biofuel project to be profitable and scalable to an industrial level [6]. This includes lipid content and quality of microalgae strains, control of biomass production at a large scale in highly variable outdoor conditions, productivity improvement, etc. To improve the use of microalgae in biofuel production, it is important to introduce techniques allowing fast, easy, and effective monitoring of intracellular and extracellular features related to their productivity. Flow CytoMetry (FCM) has, in this sense, many advantages that can contribute to successful development of microalgae-based biofuel projects given the analytical capabilities it offers.

The essence of FCM is the simultaneous measurement of different optical cell properties (light scatter and multicolor fluorescence emission). This allows an exhaustive characterization and classification of individual cells in a natural or treated sample. FCM was developed in the middle of last century and was exclusively used for medical applications. The first applications of FCM in aquatic sciences seem to have been started in the late 1970s [7], [8], [9], [10], [11]. FCM was applied to phytoplankton analysis initially for monitoring the populations in natural samples based on their auto-fluorescent properties. Use of this technique has contributed to knowledge improvement of spatial-temporary distribution of microalgal populations as well as population dynamics linked to the environment [12]. It is only recently that new application of FCM applied to microalgal biotechnology started to be developed [4], [5], [13], [14].

2 APPLICATIONS OF FCM IN BIOFUEL PROJECTS DEVELOPMENT

2.1 BIODIVERSITY MONITORING

The enormous biodiversity of microalgae makes them suitable candidates for biofuel production [15]. This natural biodiversity is estimated at 30.000 species [16] distributed in different habitats such as freshwater, seawater, brackish, wastewater, hyper saline, etc [15]. These ecosystems harbour a number of high lipid producer's microalgae strains, most of which not yet exploited.

Know microalgae population's distribution gives us a clear idea about how many and whose species can be found in a determined sampling point (period/region). According to Mutanda *et al*. [15], search, collect and identify interesting strains are first and crucial steps for a successful microalgae-based biofuels project. In this way, multi-parameter FCM analysis allows the fast and real time monitoring of microalgae populations contained in an environmental sample.

Figure 1 represents an example of intrinsic features monitoring of microalgal population within a mixed sample using FCM. In this case, the mainly used parameters are auto-fluorescence of photosynthetic pigments and cell size. Chlorophyll and phycobiliproteins (phycoerythrin and phycocyanin) that are typical of cyanobacteria (cryptophytes and rhodophytes) are the most common photosynthetic pigments of microalgae [17]. Content and composition of these pigments depend on taxon but also on environmental conditions (illumination, mineral composition of water, pH, etc.) and can be monitored by FCM [18], [19]. This allows FCM to facilitate a first and fast taxonomical identification of microalgae populations in a natural sample (Tab. 2).

Table 2. Fluorescence excitation/emission of different fluorescent pigments using FCM. LP: long pass filter [17], [20]

Pigment	Excitation	Channel (emission)	Taxonomic group
Chlorophyll	Argon 488nm	FL3 (670LP) Red	Majority of phytoplankton and microalgae
Phycoerythrin (PE)	Argon 488nm	FL2 (585/30nm) Orange	Cyanophyceae, Cryptophyceae, Rhodophyceae
Phycocyanin (PC)	Red diode 633 nm	FL4 (661/16nm) Dark red	Cyanophyceae, Cryptophyceae

Chlorophyll: Chlorophyll is present in all photoautotrophic microalgae species. After excitation with 488 nm using the argon laser, chlorophyll emits in both orange (optical filter FL2) and red (optical filter FL3) and fluorescence emission can be observed at 570 nm or 690 nm respectively [17]. The signal can be collected in FL2 and FL3 channels although FL3 channel seems to be the most used. PhycoErythrin *(PE)*: is also excited using argon laser 488 nm and emits in orange. Fluorescence is then measured at 585 nm (FL2) [17], [20]. PhycoCyanin *(PC):* generally excited at 620 nm using diode laser and emits at 640 nm (optical filter FL4) [17], [20].

Two-dimensional profiles of FL2 against FSC (Forward Scatter, indicating cell size) and FL3 against FSC are plotted and the parameter setting adjusted to monitor all chlorophyll-having populations present in the sample (Fig. 1).

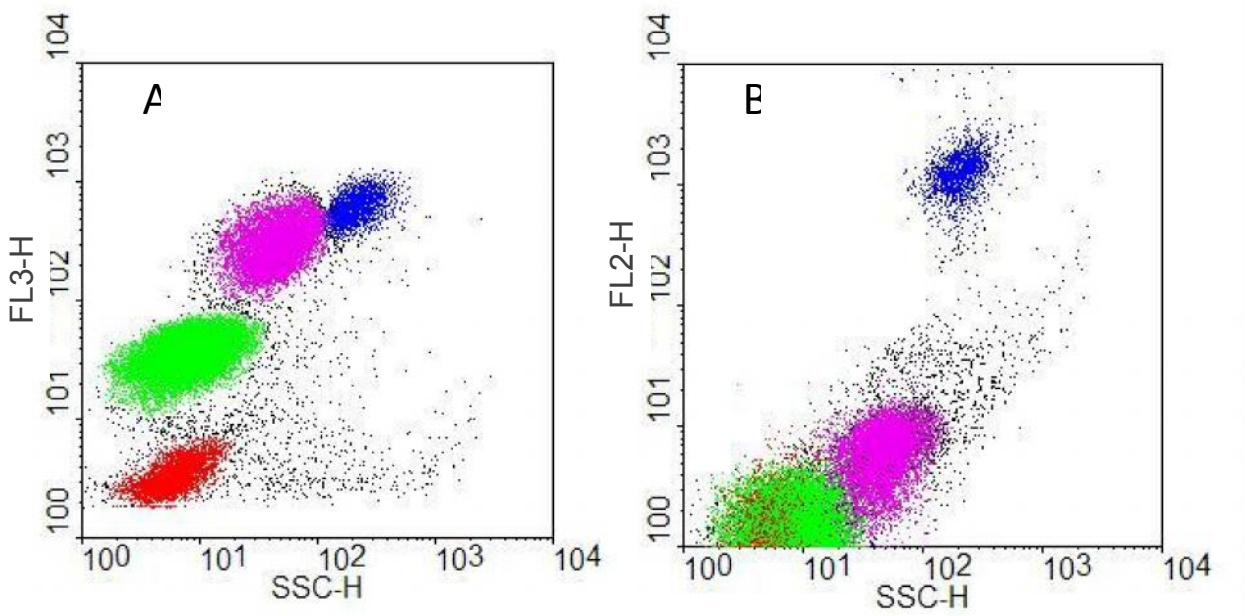

Fig. 1. Cytograms of some microalgal species analyzed using FACSCalibur cytometer, Synechococcus sp. (red), Nannochloropsis gaditana (green), Isochrysis galbana (pink) and Rhodomonas salina (blue). Dot plots presents fluorescence of chlorophyll: FL3 (A) and phycoerythrin: FL2 (B), vs celular structure. Strains belong to Culture Collection of Marine Microalgae (CCM-ICMAN-CSIC, Spain). Data not published

2.2 CELL SORTING

Once microalgae populations in an environmental sample are characterized, comes the isolation and purification of interesting populations or strains. Those steps are tedious and time-consuming. A development of fast and efficient method, for microalgae strains isolation from natural samples, becomes a mandatory given the development rate of biofuels programs worldwide.

Cell sorting coupled to FCM is based on the multi-dimensional distribution of algal populations for red fluorescence that represent essentially chlorophyll against forward-light scatter representing cell size. The first step is to identify the parameter settings that allow the best visualization of all populations contained in the fresh unfixed sample [21]. These parameters settings include voltages of FSC, SSC (Side SCatter), PE channel (FL2), chlorophyll channel (FL3), PC channel (FL4) and threshold applied to FL3 (to separate chlorophyll fluorescence from auto-fluorescence signals of other molecules). Once all sorting conditions are mastered, the population of interest is gated and sorting launched using the adequate mode. The sorting operation can take from some minutes to few hours (when the cell number of interesting populations in the sample is very low), followed by culturing individual sorted cells in culture wells using an appropriate medium. Cell sorting module present in new generation of flow cytometers offers the possibility of rapid and efficient isolation of microalgal cells from the original natural sample (Fig. 2). With this method pure populations can be isolated in few hours versus months with classical methods such as single cell isolation, dilution or micromanipulation [21], [22]. In addition, the sorter module coupled to FCM offers also the possibility to carry out an axenic sorting. To achieve an axenic sorting, the fluidic system should be thoroughly disinfected using, for instance, 70% ethanol for 20 min followed by a flushing with 0.2 µm filtered or autoclaved sheath fluid. The cell sorting is carried out using sterile sheath fluid and sterile vials to collect sorted cells.

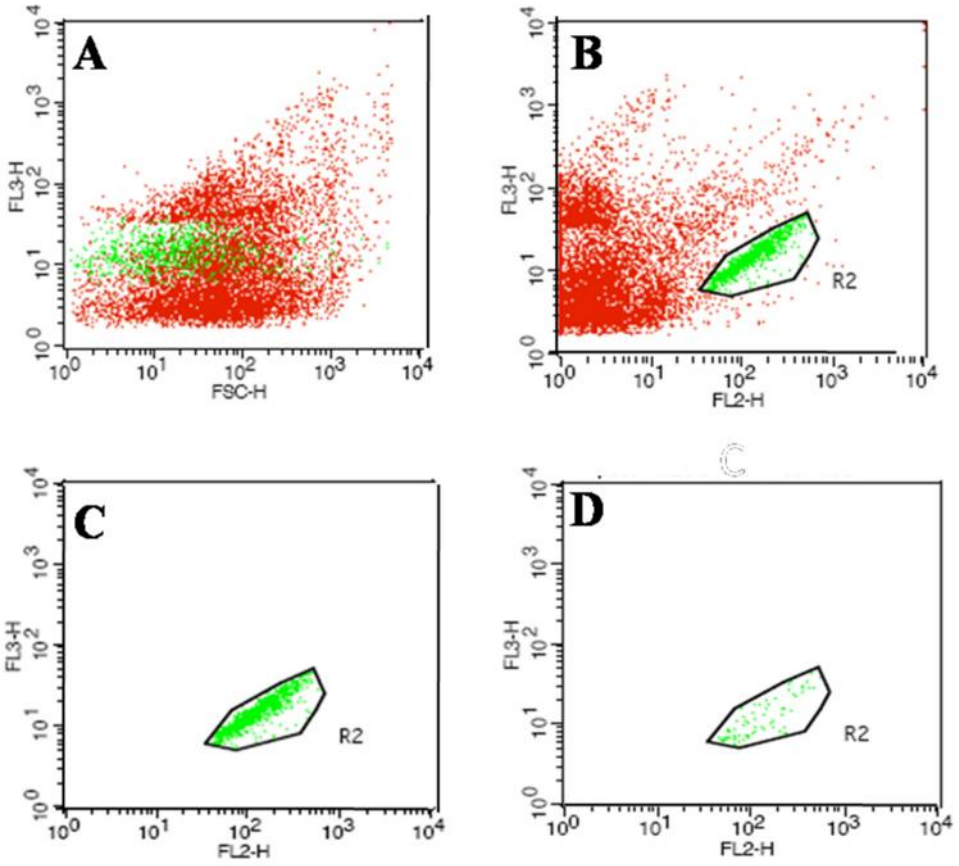

Fig. 2. Flow cytometric dot plots of sorted microalgae isolated from natural sea water from North of Morocco, using single-cells sorting mode of sorter coupled to a FACSCalibur cytometer. Experiment was carried out in our laboratory. (A) Dot plot windows based on cell size (FSC) and chlorophyll (FL3) auto-fluorescence of microalgae. (B) Chlorophyll (FL3) vs. phycoerythrin (FL2) auto-fluorescence of gated population previous to sorting. (C) Sorted cells monitoring during sorting and (D) analysis of sorted sample after centrifugation and resuspension in filtered natural sea water. Data not published

Several researchers have used FCM features for microalgae isolation in microalgae-based biodiesel project. High-lipid producing strains of *Tetraselmis* have been isolated using this method [5]. Furthermore, Doan *et al*. [4] isolated about 96 strains from costal water of Singapore for a biodiesel project.

However, a probably adverse effect of using cell sorting coupled with FCM has been described. It was reported that sorting can affect the cell viability by electrical, mechanical or optical stress (laser contact with the cells). Nevertheless, this effect is not dramatic at least in the analyzed species belonging to diatoms and green microalgae [4] when 50-75% of sorted cells remained viable. In the same manner, other researchers showed high viability (65%) of sorted *Tetraselmis* cells [5]. The viability of microalgae after sorting was significantly improved in comparison with earlier data that reported that only 20-30% of sorted cells grew successfully [23]. It's probably due to the improvement of sorting technology and also to the cell resistance against laser treatment; some species with rigid or additional cell wall like diatoms can be protected against this side effect [20].

It is important to know that for increasing the viability of sorted cells, some precautions have to be taken in consideration; firstly the sheath fluid must be appropriate for the growth of sorted microalgae, this prevents additional stress to microalgae. The laser power is another important factor affecting cell viability; it should be turned down as far as possible to minimize photo-damage of the sorted cells [20].

Another challenge of using cell sorting lies in use of natural samples containing pluricellular or filamentous microalgae communities, which cannot pass through the needle of the cytometer.

The cell-sorting coupled to FCM can also be used for re-isolation or re-purification of selected strains used for biodiesel production (i.e., when a culture becomes contaminated with bacteria, fungi or with autochthones non-lipid producing microalgae strains). Contamination occurs frequently since open culture systems are the most used for microalgae culture because of their profitability [1].

2.3 CELL COUNTING

Defining number of microalgae cells in a culture is very important during all steps of microalgae culture, mainly when the exact number of cells needs to be known. Microalgae, like most microorganisms can be counted using optical microscopy. However, the need for the use of faster and more precise systems has been noted by researchers. FCM represents an automated system, allowing precise and fast determination of microalgae number in a culture. In addition to this, it offers the possibility to distinguish smallest microalgae (picoplankton: phytoplankton <2 to 3 µm) from bacteria which is an advantage over optical microscopy. Counting microalgae, as well as other applications, is based on their auto fluorescent properties, due to photosynthetic pigments. This allows discriminating them from other microorganismds and non-living particles. For microalgae cell counting, fresh unfixed samples are preferably used, so that cells conserve their fluorescent properties. Fluorescent beads with known concentration and size can be used to measure the flow rate. This data is then used to calculate, based on flow rate, the number of fluorescent particles (microalgae) analysed by time and volume units [17]. This FCM application is a useful tool for microalgae culture destined to biofuels production, since it allows the control of microalgae growth and evolution during the culturing period.

2.4 CELL VIABILITY MONITORING

It is usually necessary to evaluate cell viability during microalgae culture for biofuel production as well as after treatments for lipids enhancement. Since microalgae are very sensitive to culture conditions, it becomes essential to develop an easy and fast method to evaluate the effect of culture conditions variations on cell viability. In open culture systems (open ponds) microalgae are exposed to medium evaporation (salinity and nutrient stress), shaking stress (agitation is generally mechanical), temperature variation between day and night (thermal stress) and other risks like biological contaminations. In closed systems like photobioreactors these risk factors are more controlled. However other problems like photodamage of cells due to illumination are more frequent. For this reason, easy and continuous control of physiological state of microalgae is very important. Specific dyes like Propidium Iodide (PI) or SYTOX Green cannot cross walls of living cells, and only once membrane integrity affected the dyes can associate with DNA and exhibit fluorescence. When a microalgae sample is stained with PI solution, it penetrates the nonviable cells, binds to DNA and after excitation with 488 nm, the fluorescence is measured at 585 nm (FL2 channel), contrary to living cells which emit no fluorescence in FL2 (Fig. 3). This method has been successfully used in various microalgae strains implied in biodiesel projects to monitor cell viability [24].

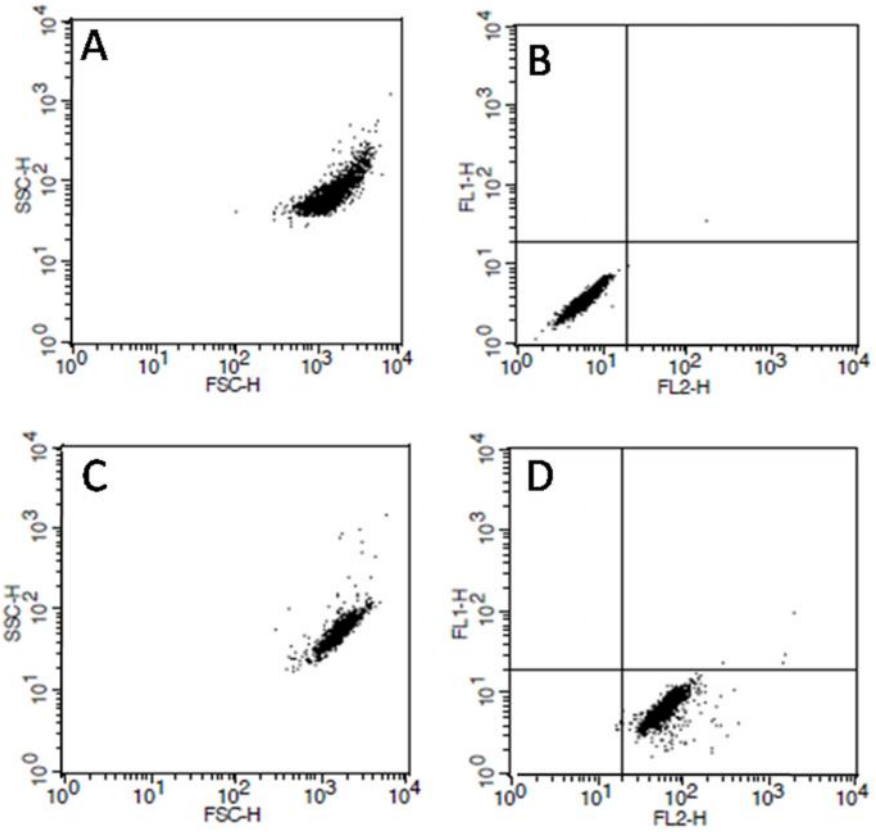

FIG 3: An example of PI staining comparison between living and died cells of Dunaliella salina isolated from saline water located in north of Morocco. Experiment was carried out in our laboratory. (A) Dot plot FSC/SSC of one population corresponding to living cells, (B) no staining of cells after incubation with PI. After heat treatment (10 min in water bath at 100°C for), no major changes in size /structure has been observed (C), but PI stained killed cells and signal is collected on FL2 (D). Data not published

2.5 INTRACELLULAR LIPIDS CONTENT

Microalgae stores lipids bodies as a form of energy reserve represented by neutral lipids, and as essential components of membranes like glycol and phospholipids. It is well established that, in most microalgae strains (mainly lipid accumulative strains), neutral lipids concentration increase significantly with culturing time and in response to culture conditions changes (nutrients starvation, light stress, CO_2 supply, etc.). This property is mainly exploited in biodiesel projects since successful and economically viable algae-based biofuels industry depends on selection of appropriate strains and their culture conditions. One of the most critical properties of strains candidates of a biofuel project are lipids content and quality. In this way, rapid and efficient methods allowing determination of lipid content are needed. In comparison with chromatographic and gravimetric techniques, that needs previous lipid extraction, fluorometric measurements are fast, cost-effective [25] and only few microliters of culture are needed *vs* milliliters to litters for lipid extraction. Thereby, use of specific dye and FCM technique offers an attractive tool. Solvatochromic dye Nile Red (NR) (9-diethylamina-5H-benzo[a]phenoxazine-5-one) stains intracellular lipids selectively allowing the quantification of neutral and polar lipids [26]. Lipid measurement of intracellular lipids using NR fluorescence is produced in highly hydrophobic environments and quenched in hydrophilic ones [27], [28]. The efficiency of NR fluorescence and the total lipid content has been shown linear [25], [27], [29].

In a flow cytometer, lipids' straining with NR is traduced to different fluorescence emissions after excitation with argon laser at 488 nm. When NR is dissolved in neutral lipids it emits an intense yellow fluorescence collected in channel FL2 (560-640 nm in a FACSCalibur™ system, Becton Dickinson Instruments, equipped with a 488 nm argon laser and 635 nm red diode laser) (Fig. 4), and when dissolved in polar lipids it exhibits red fluorescence (>650 nm) collected in channel FL3 [27], [30].

NR fluorescence showed to be an efficient high-throughput screening tool to select a favourable microalgae strain based of their intracellular lipid content, for biofuel projects [4], [13]. Different aspects can be studied: firstly kinetic of neutral lipids accumulation during the growth cycle to identify the peak of lipid production, comparison of lipid content in stationary phase between different species, and/or various growth conditions and the effect of some treatments and metabolic orientation on the lipid production. Several works have used this technique efficiently to develop biofuel projects [4], [13]. Doan *et al*. [4] screened 96 strains isolated from natural samples and found that about more than half of studied strains contained more than 25% lipids.

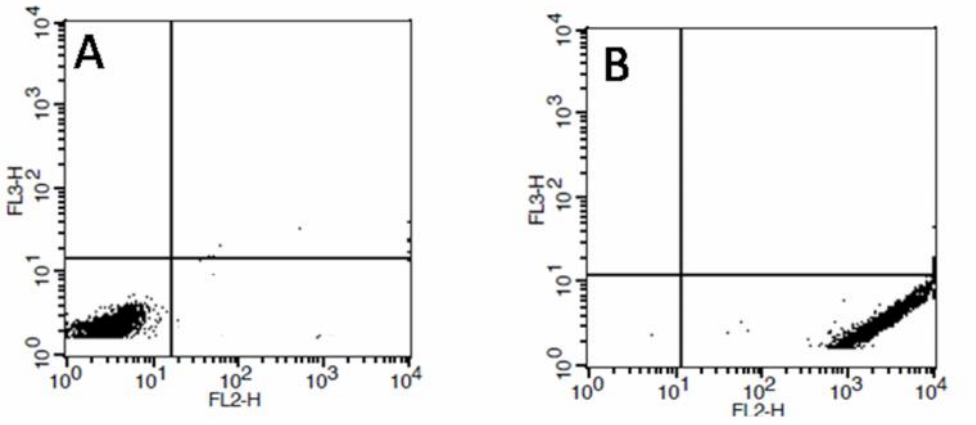

Fig. 4. NR staining dot plots of Nannochloropsis sp. isolated from North of Morocco. Experiment was carried out in our laboratory. (A) Microalgae sample before NR staining, parameter settings are adjusted to place population in negative area. (B) After NR addition, sample is incubated 2 min at 37°C and an intense signal is collected at FL2 channel indicating that this strain has an interesting neutral lipids accumulation. Data not published

The possibility to use the multi-parameter flow cytometry for fast and real time monitoring of intracellular lipids content has been studied for some microalgae species belonging to different taxonomic groups (Tab. 3).

TABLE 3: Species candidates for biodiesel project analysed by FCM after NR staining.

Microalgae	Lipid content (% of DW)	References
Skeletonema costatum	9.5	[4]
Thalassiosira sp.	17.8	[4]
Nannochloropsis sp.	40.3-44.8	[4]
Chaetoceros sp.	16.3	[4]
Achnanthes sp.	44.5	[4]
Heterosigma sp.	39.9	[4]
Tetraselmis suecica	17.7-29.9	[5]
Chlorella prothecoides	27.5	[13]
Scenedesmus obliquus	12.8	[24]
Neochloris oleoabundans	16.5-52	[24]

This technique offers multiple applications in this field such as fast, in situ and real time estimation of intracellular lipids (mainly neutral ones) between several microalgae species without the laborious stage of lipid extraction and chromatographical analysis. In addition, it gives an idea of microalgae lipid content in order to decide if the strain is interesting or not for a biodiesel project. In case of lipid rich strains, a confirmation is needed by chromatographical analysis [13]. However some species like some chlorophyceae, diatoms, etc. presents rigid cell walls that decrease the NR penetration into the cell. In these cases, when gravimetric measurements show lipid presence, improved methods have to be used for lipid analysis using FCM (Fig. 5). Permeation of cell walls using some chemical reagents is the most used methods applying

different concentrations of DMSO (5-30% v/v), ethanol or glycerol [4, 13]. Also, FCM allows easy monitoring of lipid content during the microalgal growth and the identification of maximal lipid accumulation phase, monitoring of the effect of growth conditions (culture media, light, CO_2 supply, shaking, etc.) on lipid accumulation, giving the possibility to rapidly change culturing conditions to maintain maximal productivity. When the cell lipid content information is available during the culturing process, these parameters can be controlled.

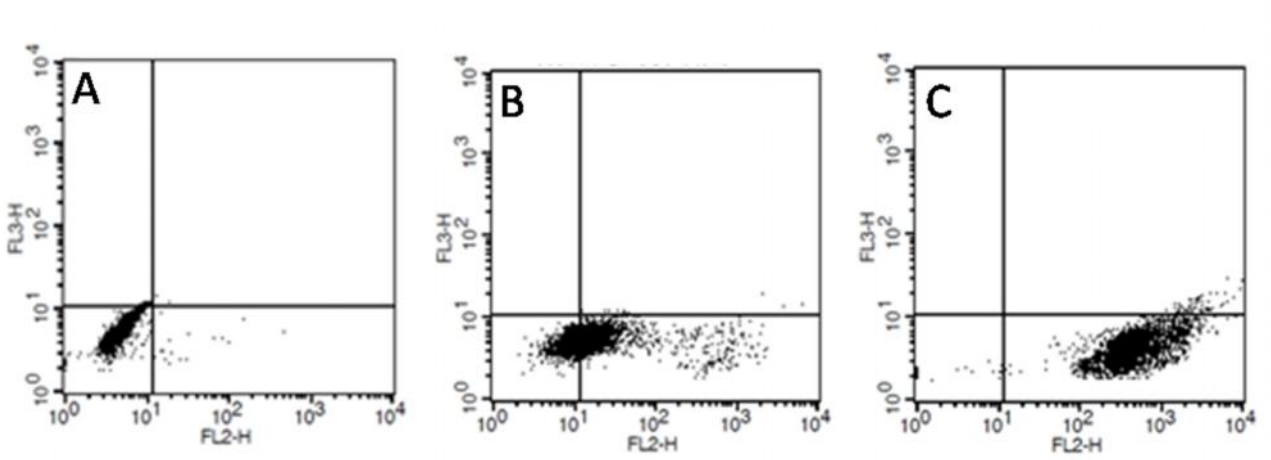

Fig. 5. Improvement of Dunaliella sp. staining efficiency using DMSO. This strain has been isolated from saline in North Morocco, and experiment was carried out in our laboratory. A: microalgae sample without staining, parameter settings are adjusted to place population in upper left area. B: Nile red addition and incubation at 37°C during 10 min. C: NR staining improvement after addition of DMSO 20% simultaneously to NR addition. Data not published

As described, FCM can be used in the most decisive steps during biodiesel project development (Fig. 6); isolation of strains from their natural habitats, screening and selection of microalgae candidates for the biodiesel production.

Both at laboratory or large scale levels, use of FCM leads to fast and efficient control of critical parameters changes such as biomass and lipids evolution in term of quality and quantity. This offers the possibility to act immediately to adjust culturing conditions, determine optimal conditions for growth and increase productivity.

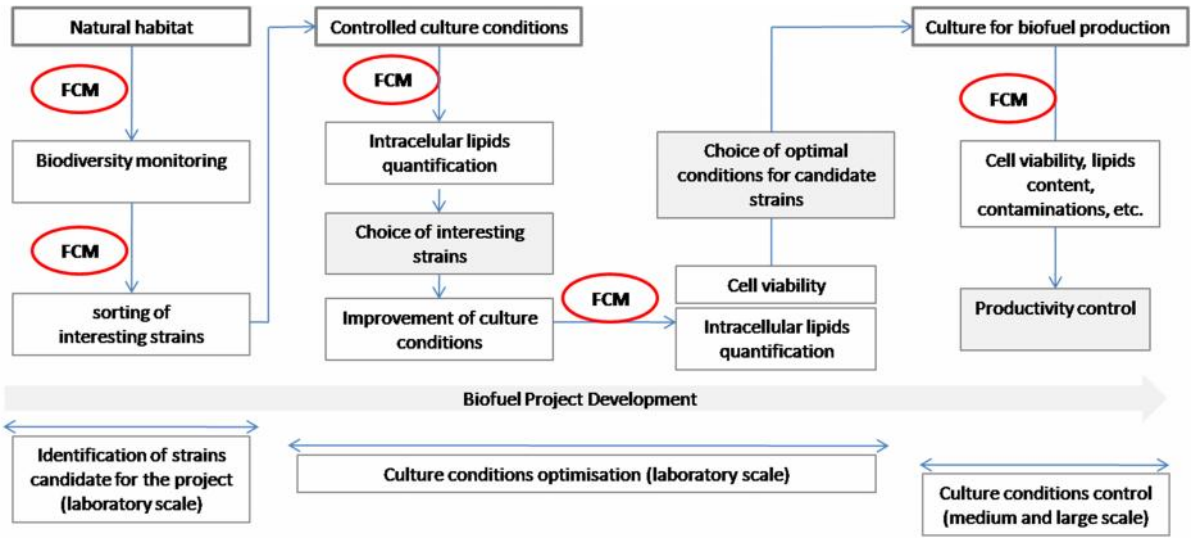

Fig. 6. Applications of FCM during steps of biodiesel project development

3 CONCLUSION

Despite the considerable increase towards the use of microalgal lipids for biodiesel production, the development of a project that is profitable at large scale still needs further investigation. In this context, many researchers are opting for development of microalagal biotechnology as a powerful way to improve productivity of microalgae. In this review, FCM is described as a multiparameter technique that can contribute efficiently in microalgal biotechnology development and represent a helpful tool for a better exploitation of microalgae as feedstock for biodiesel production.

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