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Article

Intensive Production of Carboxylic Acids Using *Cl. butyricum* in a Membrane Bioreactor (MBR)

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Abstract: This work reports of the use of a bench scale chemostat (CSTR) in continuous mode and of a pilot scale membrane bioreactor (MBR) in fed-batch mode to intensively produce acetic and butyric acids using *Cl. butyricum* grown on synthetic media. These studies were then used to perform a cost estimation study of the MBR system to assess the potential economic impact of this proposed methodology. The MBR system was found highly productive reaching 37.88 g L^{-1} h^{-1} of acetic and 14.44 g L^{-1} h^{-1} of volumetric cell productivity, favoring acetic acid production to butyric at a ratio 3:1. The cost of preparation and production of carboxylic acids using this system was found as 0.0062 £PS/kg respectively and an up to 99% carbon recovery.

Keywords: acetic acid; butyric acid; effluents; microfiltration; fermentation; MBR

1. Introduction

Uncoupling energy generation and acids production from petroleum has been classified as a high priority among Western Europe and the United States [1]. The carbon based economy, in the developed Western world is challenged due to fossil fuel scarcity and socioeconomic changes, constituting the formation of petroleum distillates, such as acetic and butyric acid from alternative sources an attractive option [2]. The production of carboxylic acids by fermentation, within the biorefinery concept ergo the biobased conversion of waste, plant biomass and other materials is becoming an effective choice applied to bench, pilot and industrial scale [3-6]. Acetic and butyric acid have numerous uses in the industry, as aroma enhances in food applications, components in cosmetics or precusors in bioplastics, their intensive production in large volumes is necessary.

Acetic acid is used as a precursor, an additive or a compound on a wide range of products, in the pharmaceutical, chemical and food industry. For example, it is used in the synthesis of acetyl cellulose and plastics, in the food industry as a food additive, an acidity regulator under the code E260, and in the printing and coating field. Another major use of acetic acid is, in the production of vinyl acetate monomer, a compound that is used for vinyl plastics, adhesives, textile finishes and latex paints and it is closely followed by acetic anhydride and ester production. Acetic anhydride is used for cellulose acetate and pharmaceuticals and plasticizer production. In households diluted

acetic acid is often used in desalting agent [7]. Butyric acid on the other hand is a popular aroma, flavouring and texture additive in the feed and food industry but also an important drug agent in the pharmaceutical industry. It can also be used as raw material for the production of biobutanol a promising biofuel of higher energy generation when burned and less volatility than ethanol [8].Acetic acid global market is expected to reach 18,296.90 kilo tons by 2023 with a financial value to surpass 8.6 billion $USD [9, 10] while butyric acid is projected to reach 329.9 $USD in value by 2022 and a 74.4 thousand tons by volume [11].

Among the carboxylic acid producing bacteria, *clostiridia* spp. have attracted significant attention in the industry and academia [12] as natural- acid producing- bioreactors. *Cl. butyricum*, commonly cultured in mesophilic, neutral to alkali, microaerophilic conditions [13] produces a mixture of acetic and butyric acid simultaneously with hydrogen and carbon dioxide. The bacterium (Fig.1 a) is a saccharolytic microorganism able to ferment a wide variety of carbohydrates (Fig.1 b) including waste streams such as confectionary waste including molasses [14-16], becoming an ideal candidate for intensive production of carboxylic acids. This however may be hindered by the toxic effect of the acids on microbial growth, limiting considerably their production.

An effective solution to this problem is the propagation and culturing of the organism in a system that would be operated either fed-batch or continuously, where simultaneously with the feed intake there would be removal of the spent effluent containing the produced acids. Such systems, usually developed in the form of upgraded batch reactors, are not currently preferred by the industry due to the complexity of operations, cost of construction and maintenance, demand for skilled operators and danger of cross contamination [17].

However, a membrane bioreactor (MBR) could offer a robust answer to this challenge. MBRs are well established systems, traditionally used in wastewater treatment as a replacement for activated sludge or flocculation processes where filtration replaces sedimentation to retain biomass within the reactor [18,19]. However they can be effectively used for intensive propagation of microorganisms, benefiting from the concept that cells can be retained in the membrane filter, forming a compressible permeable "cake" continuously generating biomass and metabolic end products. MBR systems have many advantages over continuous culture reactors or cell recycle reactors relying on sedimentation, since cell retention is controlled by a physical separation allowing application into numerous types of cells as well as versatility in operating strategies, scalability and expandability. For instance, the system could be operated fed-batch, or continuously or having another membrane component added that would allow simultaneous downstream processing since the recovered permeates would be cell free. Previous research [20,21] has shown that organic acid productivity and final biomass concentration in such a system were over 20 times greater than those for continuously stirred reactor (CSTR) operated batchwise. To the authors knowledge there are no prior reports of the growth *C. butyricum* with a membrane bioreactor.

Therefore this work reports of the use of a pilot scale MBR to intensively produce acetic and butyric acids using *Cl. butyricum* grown on optimised synthetic media. Studies were done using a bench top CSTR operated in batch and continuous mode. These studies were then used to perform, a cost estimation study of the MBR system to assess the potential economic impact of this proposed methodology.

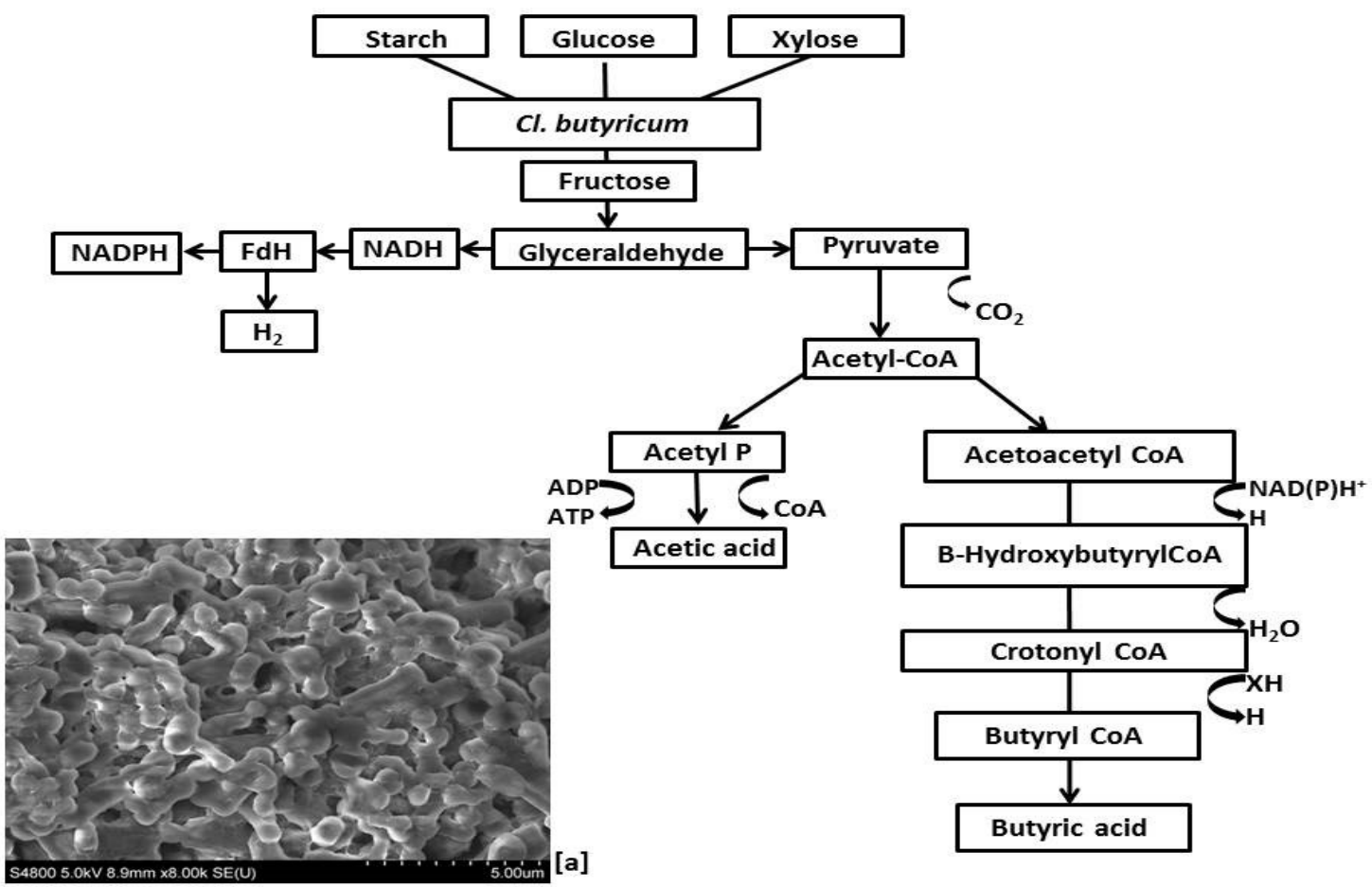

Fig. 1: a) SEM image of pure cultures of *Cl. butyricum* NCIMB 7423 used in this study (b) Biochemical pathways used during microbial metabolism of *Cl. butyricum* [51]

2.0 Materials and Methods

2.1. Materials

2.1.1. Chemicals

The yeast extract, peptone, glucose, potassium dihydrogen orthophosphate, ammonium sulphate, sodium hydroxide (NaOH) were bought from Sigma-Aldrich Chemicals, Gillingham, United Kingdom.

2.1.2. Inoculum source

Cl. butyricum NCIMB 7423 was provided in a lyophilised form by the National Collection of Industrial Food and Marine bacteria (NCIMB), Aberdeen, Scotland, United Kingdom.

2.2. Methods

2.2.1. Experimental

2.2.2. Preservation of Microorganism

The bacterium was revived twice by inoculating the selected strain into 50 ml serum vials containing optimised liquid medium (yeast extract 10 g L^{-1}, glucose 10 g L^{-1}, ammonium sulphate 5 g L^{-1} and 2.5 g L^{-1} potassium dihydrogen orthophosphate) and were statically incubated at 37 °C (Thermo Scientific Series 6000 Incubator, USA) for 24 h. Stock culture solutions of each strain were made through cryopreservation method [22,23] . For constant use, the bacterium was regularly inoculated (on a weekly basis) into 30 ml serum vials containing standard medium and were preserved at 2 °C [24].

2.2.3. Inoculum Preparation

The specified quantities of powdered materials, namely yeast extract 10 g L^{-1} , glucose 10 g L^{-1}, ammonium sulphate 5 g L^{-1} and 2.5 g L^{-1} potassium dihydrogen orthophosphate were weighted into an electronic balance (Sartorius, CP4202S, JENCONS-PLS, Germany) and they were added and mixed into an Erlenmeyer flask containing 1L of distilled water. In order to remove the existing dissolved oxygen, the medium was boiled using a Bunsen burner. Resazurin dye functioned as an anaerobiosis indicator (negative redox potential) changing its colour from deep purple to colourless. Once cooled in room temperature and achieved a pH of 6.5, the medium was dispensed into serum vials under the presence of gaseous nitrogen flow to achieve complete anaerobic conditions [25]. The medium was decanted into 40 ml aliquots, which were placed into the serum vials. The head tubes went under gaseous flow of nitrogen [26] and then sealed with rubber stoppers and aluminium Wheaton seals. The sealed tubes were secured and were autoclaved at 121 ºC for 15 min. No glucose was added prior to autoclave in the medium, as to avoid caramelization effect, but it was then added to the required concentrations to test its effect on the growth. The tubes were gently mixed in a vortex, inoculated with 4 ml inoculum size, and statically incubated at 37 °C until reaching late exponential phase (18 h of growth). The inoculum (10% v/v) was then transferred into 500 ml culture bottles containing 250 ml standard media with a nitrogen filled headspace, grown to late exponential phase. Having achieved a fully grown inoculum, it was taken into one 25.0 L culture bottles of 20.0 L working volume a nitrogen filled headspace. Inoculations were made 10% by volume. This was introduced into the MBR using a peristaltic pump and was concentrated to 15.0 L into the membrane.

2.2.4. Measurement of cellular growth and biomass

The cellular growth was measured by placing the pressure tubes into a spectrophotometer fitted with a test tube holder with 1.8 cm. light path (PU 8625 UV/VIS Philips, France) at 660 nm. Maximum specific growth rate (μ_{max}, h^{-1}) [27] and final biomass (g L^{-1}) concentration of the microbial strain were determined into basal medium under a 10 hour circle of static incubation into 32°C. To convert the optical density (O.D.) measurements into dry weight units (g L^{-1}) of the bacteria dry

weight determination assays were performed [28] resulting into of linear equation (two variables) of an intercept-slope form of $y = mX + b$ for dry weight determination where x stands for optical density units. The equations for *Cl.butyricum* is the following y=0.0959x+0.0006.

2.2.5. Analysis of end products using gas chromatography

Acetic and butyric acid were analysed using head space gas chromatography, VARIAN ProsStar GC-3800 (USA) fitted with flame ionization detector (FID) , connected with a hydrogen generator (UHP-20H NITROX, Swan Hunter, UK), with air supplied and helium used as a carrier gas, equipped with a Nukol, fused silica high-quality coated polymide capillary column 15 m x 0.32 mm I.D., 0.25 μm column, using the following protocol, of a total holding time of 15 minutes, a gas flow rate of 30ml/min and a pressure of 10 psi and an FID temperature of 220 °C as described by Sigma-Aldrich GC Supelko-Nukol columns manual.

2.2.6. Carbohydrate Consumption Rate Determination

Glucose concentration was measured using an enzymatic method using glucose oxidase (GOD) and peroxidase (POD) enzymes. The glucose (GO) determination assay kit was provided by Sigma-Aldrich, UK. The collected cultured samples were centrifuged, decanted and then microfiltered for complete removal of biomass. The integration of the colour of the solution is proportional to the concentration of glucose. The measurements were performed in bioplastic cuvettes in a spectrophotometer (Unicam, UK) at 540nm wavelength. The cuvettes, after the measurements, were cleaned with 50% ethanol solution (Aldrich Chemicals, UK) and distilled water.

2.2.7. Purity of Cultures

The purity of the cultures was tested regularly by optical microscopy (Olympus CX21, UK). Two samples were taken from each culture and colourless liquid preparations were made. The samples were checked for morphology and cell damage using phase contrast microscopy. Scanning electron microscopy (SEM) (Hitachi S4800 Scanning Electron Microscope) was also used to confirm pureness.

2.2.8. Continuously Stirred Tank Reactor (CSTR) Unit Design

Cl. butyricum were cultured (yeast extract 10 g L^{-1}, glucose 10 g L^{-1}, ammonium sulphate 5 g L^{-1} and 2.5 g L^{-1} potassium di-hydrogen orthophosphate) [29] in a 2L capacity continuously stirred tank reactor (CSTR) (Fig.2) operated batchwise and in continuous mode. The 2L round glass fermenter was set to operate at a constant temperature of 37ºC and provide pH control (6.5). pH was controlled through the use of a pump supplying alkali/acid to the culture, while the provision of gaseous nitrogen to the headspace ensured anaerobic conditions were maintained at all times. The fermenter was equipped with an autoclavable pH probe (Fisher Scientific, UK) connected to an automated pH controller (Electrolab FerMac 260, UK). This worked a pump to supply alkali/acid feed to the culture when the level of pH was detected to be below or above the set point. Agitation was provided by a magnetic stirrer coupled bar for agitation (350 rpm). The fermenter had several ports for control and sampling including a sampling and inoculation port and was sealed with silicone rubber. Anaerobic conditions were maintained through the use of a glass air lock, while gas in and gas out ports were fitted with filters (Polyvent filter, 0.2μm, Whatman Filters, United Kingdom) to prevent contamination. The temperature of the fermentation media was monitored with the use of a glass thermometer and was controlled via the use of stainless steel coils connected to a thermostatically controlled water bath [30].

During the fermentation, samples were taken aseptically on an hourly basis from the sampling port and transferred into 10 ml conical plastic tubes (Fisherbrand, UK) and centrifuged (Biofuge Stratos Sorall, Kendro Products, Germany) (4°C, 4000 g, 15 min) for complete biomass removal.

When it was determined that growth was at early death phase, the fermentation was stopped. Collected samples which had been clarified by centrifugation were then filtered (0.2 µm pore size filter).

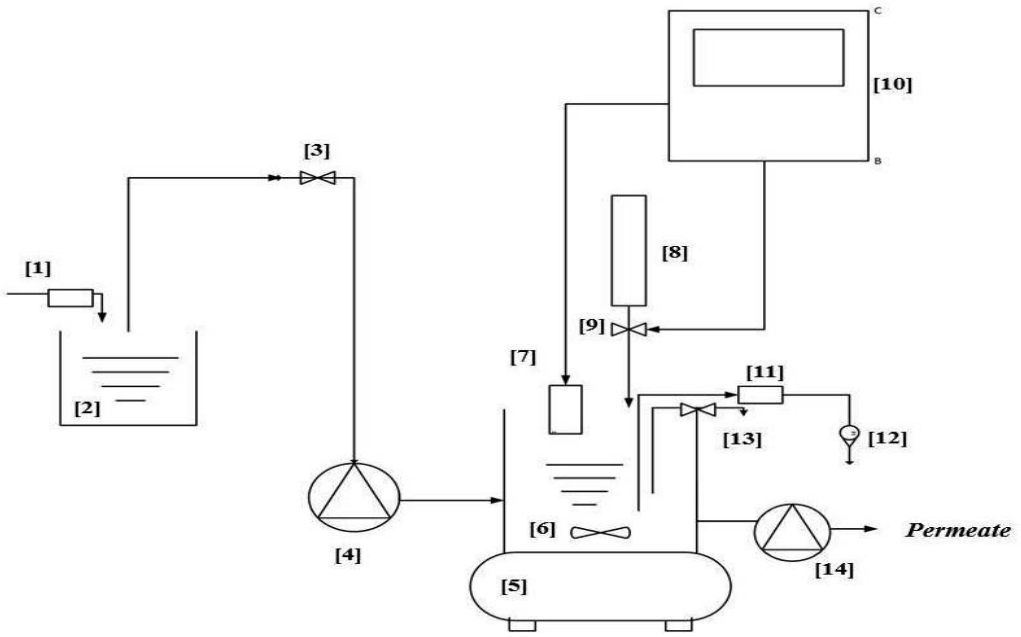

Fig. 2:Schematic diagram of the continuous culture reactor (CSTR) [1]Air Filter [2] Feed vessel [3] valve [4] Feed pump [5] Magnetic stirrer plate [6] stirring bar [7] pH probe [8] Acid reservoir [9] valve [10] pH controller [11] Ait filter [12] Rotameter [13] Sampling port [14] Effluent pump

2.2.9. Membrane Bioreactor (MBR) Unit Design

A membrane bioreactor unit (Fig.3) was developed equipped with a ceramic membrane to process the nutrient media. The unit comprised of a pressure gauge, a 5 L of 76 cm depth and 12 cm diameter, conical fermentation vessel, equipped with stainless steel coils, gas inlet and outlet, feed/inoculation port, sampling port, and drain port at the bottom. This was linked through 2 m of 1 inch stainless steel pipes arranged into two fluid loops each one driven by a centrifugal pump type Brook Crompton (Michael Smith Engineers, UK). Nutrient medium was circulated from the tank into the first pump loop which pressurised the system against a diaphragm valve (Axium Process, Hendy,Wales, UK) on the return side, which could be adjusted to control the pressure applied to the system. Within this loop an additional pump was used to feed at high flow rates the membrane and water cooled heat exchanger in series. The membrane used here was a Membralox ceramic (a-Al_2O_3) monolith microfiltration module (pore size 0.2 mm), able to withstand a pH range between 2 and 13, temperatures up to 130 °C and operating pressures between 5 to 40 psi. The membrane was fitted in stainless steel housing, commercially available by Axium (Hendy, UK). This arrangement allowed limited pressure drop in this loop, therefore the cells were effectively retained in the membrane, forming a compressible permeable cake. The effective membrane area was determined as 0.13 m^2. All the parts of the unit were connected with stainless steel hygienic clamped flanges with PTFE seals, provided by Axium Process (Hendy, Wales, UK). The MBR was sterilized at 103°C for 20 min by circulating steam through the system, while the fermentation vessel was autoclaved separately at121°C for 30 minutes . The reactor was also equipped with a level control panel, a pH controller (Model 260-Electrolab Ltd. England, 115/230V, 50/60Hz, 50W) connected to a pH probe and a peristaltic pump (Watson Marlow, England) that was used to collect the membrane permeate.

A cleaning protocol was followed to maintain the MBR performance. The membrane was then rinsed with warm water at a temperature of around 50°C. When the system appeared clean the

water was allowed to drain from the membrane and stainless steel pipe work. Next, the drain was closed and the MBR was filled with warm water. Then sodium hydroxide was added to the system to make the system pH around 11. Then the system was drained and rinsed with cold water until the pH of the system becomes 7. This was used because dishwasher cleaner is suitable for cleaning complex polyphosphates. The MBR was then operated for 30 minutes with the output from the membrane recycled into the glass vessel, and with the membrane peristaltic pump being used to back-flush the system.

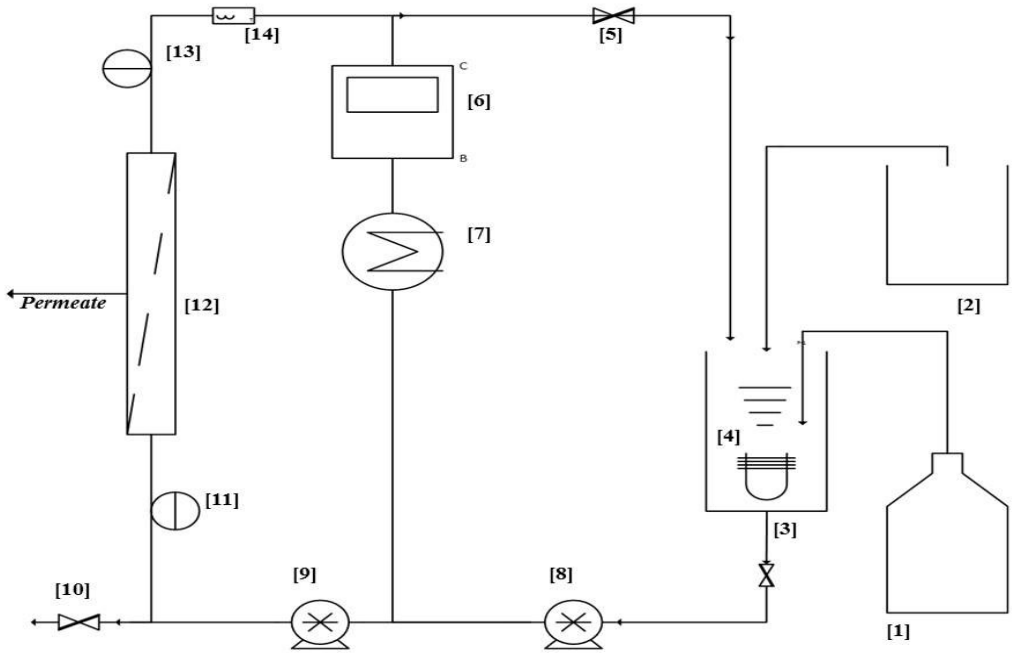

Fig. 3: Schematic diagram of the membrane bioreactor (MBR)[1]Nitrogen gas supply [2] Feed vessel [3] Reaction vessel [4] Coils [5] diaphragm valve[6] control panel [7] Heat exchanger [8] |Feed pump [9] recirculation pump [10] drain valve [11] Pressure gauge [12] Hollow fibre module [13] Pressure gauge [14] Thermocouple

2.2.10. Statistical Analysis

All the experimental data that gathered were processed through Microsoft Excel software Version 2003 using linear regression analysis. The data were analysed for accuracy and precision calculating by standard deviation, standard error, experimental error, reading error (below 5%) and regression factor. Each parameter was triplicated to obtain the average data (standard deviation of mean <5%, standard error <5%). All the numerical data were proven to be highly accurate and reproducible, having a regression factor between 0.98 and 1 and offering highly significant results.

3.0 Theoretical

3.1. Determination of Kinetic Parameters

For the estimation of the kinetic parameters used to quantify microbial growth, metabolic products generation and substrate consumption the following equations [30] were used

Overall biomass concentration (X, g L^{-1}) is being calculated according to the following formula

$$X = X_f - X_i \qquad (1)$$

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where X_f stands for final biomass concentration (g L^{-1}) at the end of the fermentation period and X_i for the initial biomass concentration (g L^{-1}) inoculated at the beginning of the process.

Specific growth rate (μ_{max}) and doubling time (DT) were calculated from the logarithmic plots of the optical density (O.D. $_{600nm}$) versus time during the exponential growth phase according to the following equations

$$\mu \ (h^{-1}) = \frac{1}{X} * \frac{dX}{dt} = \frac{d(\ln X)}{dt} = \frac{ln2}{DT} = \frac{0.693}{DT} \quad (2)$$

where DT

$$DT\ (h) = \frac{t_2 - t_1}{X} \quad (3)$$

(O.D. at 660 nm hourly basis)

The biomass yield (g L^{-1}) produced per carbohydrate consumed in a certain period can be estimated by the following equation

$$Y_{x/s} = \frac{X_2 - X_1}{S_1 - S_2} \quad (4)$$

where X_2 stands for final biomass concentration (g L^{-1}) at the end of the fermentation period and X_1 for the initial biomass concentration (g L^{-1}), S_1 stands for initial carbohydrate concentration (g L^{-1}) and S_2 for the final carbohydrate concentration (g L^{-1}).

Overall glucose consumption (ΔS) is calculated by

$$\Delta S = S_i - S_f \quad (5)$$

where S_i stands for initial substrate concentration (g L^{-1}) and S_f for final substrate concentration (g L^{-1}) measured at the end of the fermentation period.

The carboxylic acids yield (g L^{-1}) produced per carbohydrate consumed in a certain period can be estimated by the following equation

$$Y_{p/s} = \frac{P_2 - P_1}{S_1 - S_2} \quad (6)$$

where P_2 stands for final acids concentration (g L^{-1}) and P_1 for the initial acids concentration (g L^{-1}), S_1 stands for initial carbohydrate concentration (g L^{-1}) and S_2 for the final carbohydrate concentration (g L^{-1}).

Volumetric acids productivity indicating the capacity of the biomass produced per bioreactor volume to produce the metabolites of interest was calculated using the following formula

$$Volumetric\ acids\ \ productivity\ (\text{g L}^{-1}\ \text{/h}) = \frac{Final\ Concentration\ of\ acid\ in\ the\ sytem\ (^{g}/_{h})}{Total\ fermentation\ time\ (h)} \quad (7)$$

Specific acid productivity directly indicates the capacity of each cell to synthesize the metabolite of interest was calculated using the following formula

$$Specific\ acids\ productivity\ (g/g/L/h) = \frac{Total\ acid\ generated}{Integrall\ cell\ area\ (ICA)} \quad (8)$$

where

$$\text{ICA} = \frac{(final\ cell\ number - initial\ cell\ number\ ) * days\ in\ culture}{log_e\ (finall\ cell\ number\ /\ initial\ cell\ number)} \quad (9)$$

Ratio of acetic (mol/L) to butyric acid (mol/L) can be estimated by the following formula

$$\frac{C_2H_4O_2\ (acetic\ acid)}{C_3C_7COOH\ (butyric\ acid)} \quad (10)$$

Volumetric flow rate (L/h) in the reactors (continuous, MBR) is being calculated by

$$Q\left(\frac{L}{h}\right) = \frac{dV}{dt} \quad (11)$$

However several new terms were developed especially those needed to demonstrate the changes in the system. The biomass leaving the system (X_{out}) was given by

$$X_{out} = X_{in} * F * t$$

(12)

while biomass maintained in the system (X_{in}) is given by the formula:

$$X_{in} = X_0 + \frac{dx}{dt} - X_{out}$$

(13)

In the case of substrate, the substrate inflow rate (S_{in}) in the system is given by the following equation:

$$S_{in} = S_0 * F * t$$

(14)

The substrate outflow rate (S_{out}) in the system is given by the following equation:

$$S_{out} = S_{in} * F * t$$

(15)

The rate on the product maintained in the system (P_{in}) is given by the formula:

$$P_{in} = \left(Y^{p}/_{s} * (S_0 - S_{in}) - P_{out} \right)$$

(16)

The rate of product outflow (P_{out}) is given by the formula:

$$P_{out} = P_{in} * F * t$$

(17)

The dilution rate (D) is given by the equation:

$$D = \frac{F}{V}$$

(18)

Regarding the MBR

The substrate inflow rate (S_{in}) in the system is given by the following equation:

$$S_{in} = S_0 * F_s * t$$

(19)

The substrate outflow rate (S_{out}) in the system is given by the following equation:

$$S_{out} = S_{in} * \frac{dF}{dt}$$

(20)

When describing the flow rate of nutrient in the system and the flow of nutrient in the system the following equations are developed,

The flow rate of nutrient during time ($\frac{dF}{dt}$) is described by the following formula:

$$\frac{dF}{dt} = \mu * F * t$$

(21)

The flow of the substrate Fs, in the system is given by the following equation:

$$Fs = F_0 + \frac{dF}{dt}$$

(22)

The rate of product outflow (P_{out}) is given by the formula:

$$P_{out} = P_{in} * F * t$$

(23)

3.2. Cost Estimation

3.3. Process Description

The wide adoption of such an intensive bioprocessing scheme is strongly influenced by the cost efficiency of this application when compared to either the conventional methods of production or

other methods of biotechnological generation of the end products. Estimating the cost of these processes though is rather complicated as several factors have to be taken into careful consideration, such as capital cost related to manufacturing and maintenance of the system and relevant equipment, labour costs, energy consumption and transportation of waste.

To investigate the feasibility of using a membrane bioreactor for industrially relevant fermentations, a costing study was conducted using the factorial method of cost estimation [31,32]. The study was based on the development of an industrial scale reactor. The unit would be able to receive 10 m^3/h of liquid nutrient media. All costs are given in 2018 British pound £. Where necessary costs were converted using the Marshall Swift Index (MSI) for equipment, the Producers Price Index (PPI) and the Consumer Price Index (CPI) for miscellaneous costs. The location of both units is assumed to be in the United Kingdom.

3. 4. Design and Cost of the Units

The basis of the analysis is the treatment on a daily basis of 220 m^3 per day of formulated nutrient broth made of powdered materials and deionised water. Since the membrane system was designed to deal with 10 m^3/h, 2 hours per day are assigned to cleaning and maintenance of the units. The unit is made of stainless steel 304 with dairy fittings connected to a bubble column reactor. The ultrafiltration membrane selected of 0.15 m x 1.83 m, a total of 12.7 m^2 surface area per module of 15 hollow fiber modules of a total surface area of 180 m^2 [33,34]. All components of the unit are commercially available from numerous companies in the United Kingdom and worldwide. The unit is equipped with pressure gauges, pH and temperature meters and level gauges with the equipment used being commercially available.

3.5. General Economic Parameters

Operating costs can be broken into several main categories including equipment, labour, maintenance, utilities and raw materials. The total investment cost (TIC, $) is calculated by adding fixed capital (FC, £) and working capital (WC, £) [31, 32,35]

$$TIC = FC + WC$$

The direct production costs (DPC, £) or annual operating cost (AOC, £) are calculated by adding variable costs (VC, £) and fixed costs (WC, £)

$$DPC = AOC = VC + WC$$

The production cost (PC, £/kg) is calculated by annual operating cost (AOC, $/year) divided by the annual production rate (APR, kg/year).

$$PC\left(\frac{£}{kg}\right) = \frac{AOC, £/year}{APR, kg/year}$$

4.0 Results and Discussion

4.1 Growth on a Continuously Stirred Tank Reactor (CSTR) on Continuous Mode

The continuous culture of *Cl. butyricum* was conducted in a chemostat, in other words, fresh medium [29] was continuously added, while the cultured liquid was continuously removed to maintain the culture volume constant. The propagation was initiated as a batch culture, when the growth reached early stationary phase, prior to the nutrient becoming a limiting factor, fresh feed was introduced and the system was switched to continuous culture.

The continuous experiments required long periods of operation (weeks) during which the bioreactor was continuously flushed with nitrogen at a pressure of 0.5 bar, and operated at 37°C and pH 6.5. Glucose was selected as the main carbohydrate due to its higher production levels than those of xylose and starch. Glucose concentrations in the feed nutrient broth were 5, 10, 15, 20, and 28 g L^{-1}. The feed nutrient broth was introduced to the CSTR at 5 different flow rates of 0.070, 0.14, 0.20, 0.25 and 0.28 L h^{-1}, offering 5 dilution rates (D) varying between 0.058 h^{-1} to 0.23 h^{-1} (Table 1).

Five or more data points were selected to calculate the average of biomass for each dilution rate for the period during which the biomass was in steady state (Fig .4). The level of biomass produced for concentrations of 15, 20 and 28 g L^{-1} glucose are initially increasing then gradually decreasing as

glucose is spent by the bacterium's metabolism (Table 1). For 5g L^{-1}, in contrast with 10 g L^{-1}, the concentration biomass decreases while the dilution rates increased. Probably, in this case, there is poor uptake of glucose to support growth. At 0.058 h^{-1} the amount of biomass is 0.34 g L^{-1}, dropping to 0.22 g L^{-1} at 0.23 h^{-1}. When glucose concentration in the feed was raised to 15 g L^{-1} biomass increased from 0.50 g L^{-1} at 0.058 h^{-1} to 1.40 g L^{-1} at 0.21 h^{-1}, while slightly decreased at 1.04 g L^{-1} at a dilution rate of 0.23 h^{-1}. A similar pattern was developed when glucose concentration was elevated at 28 g L^{-1}.Interestingly, at a 10 g L^{-1} of glucose concentration in the feed, the results are different to the glucose concentrations adopted, as continuously increasing pattern is developed.

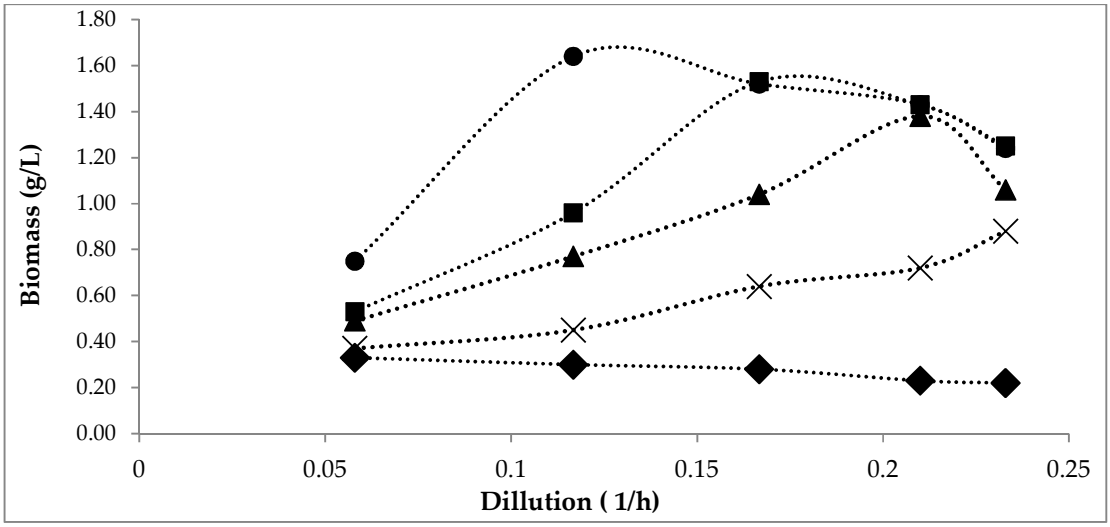

Fig. 4 : The growth of *Cl.butyricum* on glucose media at three concentrations of glucose on steady state (◆) for 5 g L^{-1} feed,(**x**) 10 g L^{-1} feed (▲) 15 g L^{-1} feed , (■) 20 g L^{-1} feed and (•) 28 g L^{-1} feed

4.2. Product Formation on a Continuously Stirred Tank Reactor (CSTR) on Continuous Mode

Samples of the propagation of *Cl. butyricum* in the chemostat were collected and analysed, to estimate the systems productivity, volumetric and specific regarding the generation of carboxylic acids. *Cl. butyricum* is a mixed acid producer, simultaneously with other metabolic products as solvents i.e. ethanol, butanol or vicinal diaketones, glycerol or 1.3 propandiol [35-37]. The patterns of acids production exhibit certain similarities with acetic and butyric acid at high glucose concentration (15 to 28 g L^{-1}), being initially increased at low dilution rates followed by dramatic decreases as the dilution rate is increased (Table 1). The highest levels of butyrate production are achieve at 28 g L^{-1}, at a D of 0.058 h^{-1} while acetic acid highest concentration was found at 15 g L^{-1} at a D of 0.21 h^{-1}. Similarly to the pattern of biomass generation at 10 g L^{-1} of glucose concentration in the feed , the concentration of acetic and butyric acid gradually rise simultaneously (Table 1), from 5.87 to 7.70 g L^{-1} for acetic acid and 2.05 to 3.50 g L^{-1} for butyric acid.

However, in the minimum concentration of glucose in the feed that was used, 5 g L^{-1}, low concentrations for both acids were observed with decreasing pattern formed over the whole range of dilution rates. Carboxylic acids production at these low glucose fermentations were markedly different showing a marked shift towards acetate production over butyrate. Glucose uptake kinetics are poor and the subsequently rates of carbon and electron flow pathways from glucose to butyrate are low. It can be assumed that product formation was directly related to the glucose consumed; this is clearly shown across the range of concentrations tested, in particular at 20 and 28 g L^{-1} glucose concentration in the feed. The generation of acetic acid is favoured in this culturing system, with the highest ratio favouring acetic acid production being found at a 5g L^{-1} initial glucose concentration,

30:1, while at 10 and 15 g L^{-1} initial of glucose the ratio becomes 4:1 or 3:1, and 3:1 or 2:1 at the highest concentrations of 20 and 28 g L^{-1} (Table 1). The ratios remain stable over the range of dilution rates but were altered by the amount of glucose present in the fermentation. Acetic acid production reached its highest at 15 g L^{-1} of glucose initial concentration at a D of 0.23 h^{-1}, followed by 10 g L^{-1} at the same dilution rate. At elevated concentration of the feed substrate, acetic acid production is reduced possibly due to the formation of other products besides butyric acid, like solvents or hydrogen [36-38]. This would be consistent with the observations associated with alkali consumption and the poor carbon recoveries observed at high sugar concentrations.

Another aspect of evaluating the efficiency for such an intensive culturing system is to examine the yield generation in terms of carbon output and recovery in relation to carbon input. As carbon input the carbon molecules contained in the carbohydrate and nitrogen feed source, in this case glucose and yeast extract, were considered while cellular biomass, carbon dioxide and acetic and butyric acid are held as carbon output. These calculation were made taking into consideration the following assumptions: a) cell biomass ($CH_{1.77}O_{0.49}N_{0.24}$) contains 50 wt% carbon [39] , b) yeast extract which is mainly comprised of the water-soluble components of the yeast cell, the composition of which is primarily amino acids, peptides, carbohydrates, and salt, is assumed containing at least 50 wt% carbon [40,41] that is consumed fully c) during *Cl. butyricum* metabolism , for each mole of acetic acid produced, 1 mole of CO_2 is generated, while for each mole of butyric acid 2 moles of CO_2 are produced (Fig. 1 b.).

High percentages of carbon recoveries (above 60 %) were observed in initial glucose concentrations of 10 and 15 g L^{-1} (Table 1) with high production of acids and biomass being observed, suggesting that the system is taking advantage of *Cl. butyricum* metabolism fully promoting intensive production of acids. On the other hand, when higher concentrations of glucose were introduced the carbon recovery percentage is not as high (above 40%), while the generation of microbial biomass is favoured over the acids production, especially in the case of butyric acid (Table 1). The rate of glucose feed has a significant effect on the carbon balance, suggesting that the carbon and electron flow pathways within the cells are altered allowing new product formation and a shift of metabolism toward more reduced end product in butyrate. This suggests especially that the capacity of electron flow to hydrogen becomes saturated or altered so that additional reducing electron equivalent are passed to butyrate so reducing the proportion of the oxidise product, acetate. Further study would be required to evaluate and confirm these interesting possibilities, for example the measurement of hydrogen and the determination of other potential end products that are also most certainly formed in cultures that contain high sugar concentration (i.e. >20 g L^{-1}). Possibly, other neutral non-volatile solvent materials might also be formed, explaining the lower carbon recovery.

When the initial glucose feed was set at 5 g L^{-1}, the lowest total carbon recovery is observed, both in terms of acids and biomass generation. Due to the overall elevated generation of carboxylic acids, it is being assumed that the carbon deriving from yeast extract is being completely utilised, while glucose is directed apart of carboxylic acids production towards biomass generation and systems maintenance.

However, most notable was the relative sugar uptake of systems which showed that the affinity for glucose uptake was related to glucose concentration with the affinity increasing with increasing sugar concentrations.

Initial Glucose Concentration (Si, g L^{-1})	Dilution rate (D, h^{-1})	Glucose Spent (ΔS, g L^{-1})	Biomass (X, g L^{-1})	Carboxylic acids concentration (g L^{-1})		Carboxylic acids volumetric productivity ($g^{-1}L^{-1}h^{-1}$)		Carboxylic acids specific cell productivity ($g^{-1}g^{-1}L^{-1}h^{-1}$)		Biomass Yield ($Y_{x/s}$)	Carboxylic acids yield ($Y_{p/s}$)	Total Carbon Recovery (%)
				Acetic acid	Butyric acid	Acetic acid	Butyric acid	Acetic acid	Butyric acid			
5	0.058	1.4	0.34	3.49	0.18	0.2	0.01	0.59	0.03	0.24	0.73	33.70
	0.117	1.2	0.3	3.13	0.18	0.37	0.02	1.23	0.07	0.25	0.66	35.32
	0.167	1.1	0.29	2.83	0.18	0.47	0.03	1.62	0.10	0.26	0.60	36.87
	0.21	1.0	0.23	2.71	0.18	0.57	0.04	2.48	0.17	0.23	0.58	40.11
	0.23	0.8	0.22	2.65	0.09	0.61	0.02	2.77	0.09	0.28	0.55	43.74
10	0.058	5.1	0.48	5.87	2.05	0.34	0.12	0.71	0.25	0.09	0.79	77.16
	0.117	5.58	0.56	6.02	2.62	0.7	0.31	1.25	0.55	0.1	0.86	83.45
	0.167	7.19	0.9	6.36	2.91	1.06	0.49	1.18	0.54	0.13	0.93	83.46
	0.21	7.86	1.08	6.93	3.05	1.46	0.64	1.35	0.59	0.14	1.00	86.78
	0.23	8.1	1.25	7.7	3.5	1.77	0.81	1.42	0.65	0.15	1.12	96.91
15	0.058	8.41	0.5	6.16	2.09	0.36	0.12	0.72	0.24	0.06	0.55	68.06
	0.117	8.96	0.78	6.78	2.93	0.79	0.34	1.01	0.44	0.09	0.65	80.42
	0.167	9.45	1.06	7.08	3.61	1.18	0.6	1.11	0.57	0.11	0.71	88.64
	0.21	10.2	1.4	7.78	4.46	1.63	0.94	1.16	0.67	0.14	0.82	99.87
	0.23	10.0	1.05	7.39	3.78	1.7	0.87	1.62	0.83	0.11	0.74	90.18
20	0.058	13.5	0.54	7.04	4.67	0.41	0.27	0.76	0.50	0.04	0.59	80.40
	0.117	13.79	0.98	8.24	5.2	0.96	0.61	0.98	0.62	0.07	0.67	92.61
	0.167	14.04	1.56	5.66	3.33	0.95	0.56	0.61	0.36	0.11	0.45	65.24
	0.21	13.68	1.45	4.47	2.31	0.94	0.49	0.65	0.34	0.11	0.34	50.80
	0.23	13.32	1.27	3.77	1.92	0.87	0.44	0.69	0.35	0.1	0.28	43.44
28	0.058	17.65	0.77	7.76	5.11	0.45	0.3	0.58	0.39	0.04	0.46	80.00
	0.117	14.49	1.67	6.98	5.03	0.82	0.59	0.49	0.35	0.12	0.43	78.25
	0.167	13.05	1.55	5.23	3.53	0.87	0.59	0.56	0.38	0.12	0.31	61.93
	0.21	12.49	1.46	4.81	2.82	1.01	0.59	0.69	0.40	0.12	0.27	55.07
	0.23	11.03	1.27	3.85	1.94	0.89	0.45	0.70	0.35	0.12	0.21	41.39

Table 1: Performance of *Cl.butyricum* (carboxylic acids productivity, biomass generation , glucose consumption , carbon recovery) in a continuous culture (chemostat) system

4.3 Growth on a Membrane Bioreactor (MBR) on Fed-Batch Mode with Varying Permeation rates

The application of membrane separation technology to biological process systems, namely biological cell reactors has attracted many researchers as a method of process intensification, thereby encouraging the process to operate faster with an increase in productivity [42]. Previous researchers have reported that MBR have been applied in high concentration cell cultivation to produce organic products [43, 44].

Despite the continuous culturing system being considered of the most productive forms of microbial propagation regarding metabolites and biomass generation, highly improved results have been developed when an MBR operated in fed-batch or continuous mode has been used. This is a highly intensive form of growth and product formation as the cells are retained within the system whilst the spent medium is removed as permeates through the membrane. Consequently, the rate feed flow rate (L h^{-1}) can be very high, well above that observed in continuous culture and as such allowing an intensive carboxylic acids production process. Using this approach, toxic end-products including end products are removed and potentially boosting the kinetic performance of the cells. In addition the product stream is cell free and highly amenable to further processing within the scope of product separation and purification. The performance of the reactor was assessed using a range of glucose concentrations (5-15 g L^{-1}), and a substrate supplementation strategy at every 4 hours, of varying flow rates flow rates (4-32 L h^{-1}) in the feed (Table 2). The pH and temperature were set at pH 6.5 and 37°C and maintained during the process.

The system was inoculated with a 20 L volume of fully grown (mid stationary phase) of *Cl.butyricum*, followed by the initiation of the feeding strategy. The cells were retained in the system while the feed rate was increased in proportion to the cell concentration increase with the aim of maintaining the medium in excess. The feed rate was initially set at 4 L h^{-1}, then at every doubling of the biomass was doubled (Table 2). During these trials samples were taken periodically for analysis of cell concentration, substrate consumption and product generation.

Fig.5 I, II, III demonstrates the relationship between biomass development and glucose consumption in the MBR for three different feed glucose concentrations, 5 , 10 and 15 g L^{-1}) and four permeation rates (Zones A-D, 4, 8, 16, 32 L h^{-1}) at each feed concentration. For all the concentrations; the consumption of glucose increased whilst, at the same time, the concentration of biomass increased.

Figure 5 (I) shows the results for a 5 g L^{-1} feed glucose concentration. In zone A, with a permeation rate of 4 L h^{-1}, the amount of biomass almost doubles from 0.367 g L^{-1} to 0.489 g L^{-1}, for glucose it decreased from 4.87 g L^{-1} to 3.88 g L^{-1}. In zone B, (permeation rate 8 L h^{-1}), the concentration of biomass increases to 0.739 g L^{-1}, approximately two times, while the concentration of glucose dropped to 2.63 g L^{-1}. In zone C (permeation rate 16 L h^{-1}), the biomass concentration gradually increased to 1.66 g L^{-1} while the glucose concentration decreased to 1.07 g L^{-1}. Subsequently, zone D, with a permeation rate of 32 L h^{-1}shows the biomass concentration increased slowly to 1.93 g L^{-1} and glucose concentration was 0.92 g L^{-1}. When glucose concentration is doubled (10 g L^{-1}, Fig. 5 II) zone A, biomass concentration increases slowly from 0.38 g L^{-1} to 0.83 g L^{-1} and glucose consumption decreased from 9.76 g L^{-1} to 4.51 g L^{-1}. Then, in zone B, biomass increases rapidly to 2.56 g L^{-1}, approximately a three and half times increase, while glucose concentration was 1.12 g L^{-1}. In zone C, biomass still increased to 3.77 g L^{-1} with a glucose concentration of 0.14 g L^{-1}. Finally in zone D, the biomass continuously increased to 4.82 g L^{-1} with glucose concentration of 0.15g L^{-1}. Finally in Fig. 5 III (15 g L^{-1} feed glucose) the following results are observed, in zone A the biomass started with a concentration 0.38 g L^{-1}, increasing to 1.56 g L^{-1}, while reducing the glucose concentration from 13.50 g L^{-1} to 3.71 g L^{-1} . In zone B, the biomass concentration increased substantially to 3.26 g L^{-1} with glucose concentration being reduced to 0.39 g L^{-1}. In zone C, biomass increased gradually to 4.78g L^{-1} and the concentration of glucose dropped to 0.09 g L^{-1}. Then, in zone D the biomass slowly increased to 5.90 g L^{-1} and with the concentration of glucose at 0.028g L^{-1}.

The highest growth rate (μ_{max}) was achieved at 15 g L^{-1} glucose concentration (Table 2). It started with a μ_{max} of 0.24 h^{-1} at a 4 L h^{-1} permeation rate, increasing dramatically to 0.75 h^{-1} at 8 L h^{-1}, then

decreasing gradually at 16 L h^{-1} to 0.55 h^{-1} and at 32 L h^{-1}flow rate, decreased to 0.43 h^{-1}. This is a common trend in all experiments the μ initially increases and then reduces at the higher rate feed rates.

This can be attributed mainly to two factors, the first being substrate limitation (Fig 5 I, II, III zone C and D) and the second is the relatively high biomass concentrations observed towards the end of the fermentations. At high biomass concentrations, the maintenance component of glucose consumption which is dependent on the biomass concentration means that the proportion of carbon utilised for maintenance increases. The implications of this are that growth rates will decline and so will cell yields on glucose.

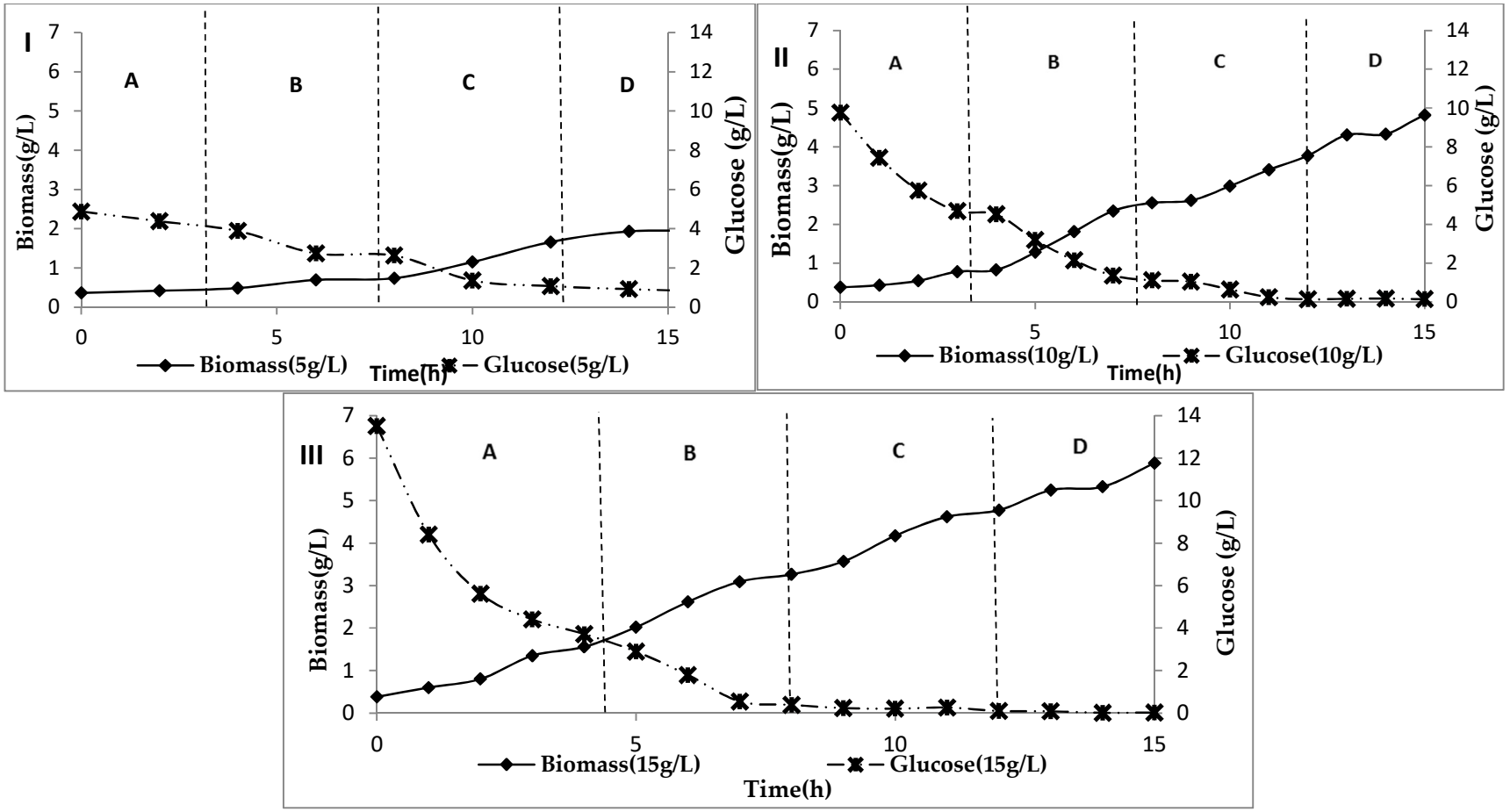

Fig. 5 (I, II, III): The growth (◆) and consumption of feed(✱) of *Cl.butyricum* on glucose media at three concentrations of glucose. (I) for 5 g L^{-1} feed,(II) 10 g L^{-1} feed and (III) 15 g L^{-1}, at permeation rates: A: 4 L h^{-1}, B: 8 L h^{-1}, C:16 L h^{-1}, D:32 L h^{-1}

Initial Glucose Concentration (Si, g L^{-1})	Flow rate (L h^{-1})	Glucose Spent (ΔS, g L^{-1})	μ_{max}(h^{-1})	Doubling time (h)	Acetic acid volumetric productivity ($g^{-1}L^{-1}h^{-1}$)	Butyric acid specific cell productivity ($g^{-1}L^{-1}h^{-1}$)	Biomass (X, g L^{-1})	Acetic acid specific cell productivity ($g^{-1}g^{-1}L^{-1}h^{-1}$)	Butyric acid specific cell productivity ($g^{-1}g^{-1}L^{-1}h^{-1}$)	Total Carbon Recovery (%)
5	4	2.26	0.07	13.56	1.65	0.09	0.74	2.23	0.12	31.29
	8	4.17	0.18	5.51	6.26	0.29	2.25	2.78	0.13	58.04
	16	4.72	0.22	4.56	14.33	0.63	4.23	3.39	0.15	77.65
	32	4.85	0.16	6.42	29.68	0.85	5.68	5.23	0.15	84.31
10	4	5.32	0.18	5.56	3.56	0.92	0.83	4.29	1.11	61.69
	8	8.88	0.52	2.03	10.42	3.14	2.62	3.98	1.2	84.31
	16	9.35	0.50	1.94	18.68	6.92	4.33	4.31	1.6	90.03
	32	9.81	0.40	2.54	37.88	14.34	5.76	6.46	2.45	99.46
15	4	10.59	0.25	4.10	4.16	1.58	1.35	4.48	1.17	64.23
	8	14.45	0.75	1.33	14.37	3.21	2.62	5.48	1.23	84.53
	16	14.74	0.55	1.83	28.29	6.49	4.17	6.78	1.56	90.11
	32	14.97	0.43	2.35	57.19	14.51	5.88	9.73	2.47	99.71

Table 2: Performance of *Cl.butyricum* (carboxylic acids productivity, biomass generation , glucose consumption , carbon recovery) in a MBR fed-batch culture system

4.4. Product Formation on a Membrane Bioreactor (MBR) on Fed Batch Mode with Varying Permeation rates

Organic acids production namely acetic and butyric acid were monitored during the MBR fermentations, while the acids produced are removed in the permeate, however, there are still considerable amounts of organic acids present in the reactor during the operation. Fig.6 I, II, III demonstrates the production of acetic and butyric acid at different permeation rates (4, 8,16, 32 L h^{-1}) using 5, 10, 15 g L^{-1} glucose feed. It can be understood that for every increase of permeation rate that the production of acetic and butyric acids increased. In 5 g L^{-1} glucose feed (Fig 6. I) zone A, acetic acid production, with a permeation rate of 4 L h^{-1}, started at 0.14 g L^{-1} at 0 h and increased to 0.85 g L^{-1}. The permeate flow rate was then increased to 8 L h^{-1}, zone B. Here, there was an immediate increase in the concentration of acetic acid to 2.27 g L^{-1} while zone C, there was further increase to 3.70 g L^{-1}. Then in the zone D acetic acid production was 4.20 g L^{-1}. On the other hand, butyric acid concentrations did not change substantially over the range of permeation rates. For this concentration, the amount of butyric acid was measured at levels of 0.11 g L^{-1} to 0.14 g L^{-1} over the period of 0 h to 15 h. This could be explained, suggesting that the conditions within the MBR favour acetic acid production over butyric acid production. When 10 g L^{-1} glucose (Fig 6. II) is used in the feed rate produces higher concentrations in a rapid rate of acetic and butyric acids when compared to the 5 g L^{-1}. Acetic acid concentration started at 1.96 g L^{-1}, at 0h, and increased to 6.76 g L^{-1} a 4 L h^{-1} permeation rate (zone A). In zone B, the concentration of acetic acid gradually increased to 8.19 g L^{-1}, then, for zone C (16 L h^{-1}) to 9.34 g L^{-1}. At a permeation rate of 32 L h^{-1} (zone D), in acetic acid production reaches 9.82 g L^{-1}. Butyric acid production shares a similar trend increasing gradually at each permeation zone, zone A, 0.18 g L^{-1} at 0 h to 2.14 g L^{-1}, in zone B to 2.19 g L^{-1}, in zone C to 2.28 g L^{-1}. Lastly, in zone D, butyric acid concentration levels reached 2.49 g L^{-1} by 15h. When the feed concentration rises to 15 g L^{-1} (Fig 6 III) production of acetic and butyric acid was faster and higher compared with the two previous feed concentrations. In zone A, (4 L h^{-1} permeation rate), the concentration of acetic acid started at 1.96 g L^{-1} and increased to 8.17 g L^{-1}, at zone B, acetic acid concentration increase climbs to 15.81 g L^{-1} while in zones C and D the concentration was stable reaching 16.35 g L^{-1}. Again a similar trend is observed for butyric acid, in zone A, it started with 0.187 g L^{-1} of butyric acid concentration and increased to 2.34 g L^{-1}. Then, the concentration increases further in zone B, (permeation rate at 8 L h^{-1}) the concentration of butyric acid increased to 3.20 g L^{-1}. After this (in zone C and D) the amount of butyric acid increased slightly to 3.30 g L^{-1}. Add comments on the yields of carb acids and biomass

In the MBR culturing system the ratio between acetic to butyric acid is favoured towards acetic acid, with the highest ratio being present at 5 g L^{-1} and increasing with increasing permeation rates. The ratio, at 4 L h^{-1} was approximately 18: 1 mol, this is because cell concentration increases as the permeation rate increases, but the source of carbohydrate was limited to sustain the production of both acids. At 8 L h^{-1} the ratio increases to 30:1 favouring acetic acid, while at 16 g L^{-1} and 32 g L^{-1} reaches 33:11 and 34:1 mol acetic to butyric acid.

At about 10 g L^{-1} the ratio of acetic acid to butyric acid, still favours acetic acid, decreased as the permeation rates increased, possibly because the source of carbohydrate was adequate for butyric acid production since its production increased. The ratio, at 4 L h^{-1} reaches 3.8: 1 mol, 8 L h^{-1} rises to 4.91:1, while at 16 g L^{-1} and 32 g L^{-1} drops to 3.1:11 and 3:1 mol acetic to butyric acid. Then at 15 g L^{-1} glucose the ratio increased in the 4 L h^{-1}permeation rate, thereafter becoming similar to that of other permeation rates. When the permeation rate is at 4 L h^{-1} the ratio became 2.64: 1 mol, 8 L h^{-1} rises to 3.31:1, while at 16 g L^{-1} and 32 g L^{-1} rises to 4.93:1 and 4.95:1 mol acetic to butyric acid.

In terms of carbon recovery supplemented in the feed (Table 2) , at 5 g L^{-1} initial glucose concentration, only 31.29% was recovered at a 4 L h^{-1} permeate rate increased to 84.3 % at 32 L h^{-1} permeate rate. At 10g L^{-1} of initial glucose concentration, 61.7 % was recovered at 4 L h^{-1} permeation rate and then increased to 99.5 % at a 32 L h^{-1} rate. Using 15 g L^{-1} initial glucose, 64.2% of the carbon was recovered at 4 L $h^{-1}$and this increased to 99.7 % carbon recovery at 32 L h^{-1}. Carbon is utilised largely for acids generation, mainly acetic acid, during the microbial metabolism while an amount is used for cellular biomass generation and maintenance. Lower permeation rates lead to lower carbon

recovery, while higher permeation rate combined with higher carbohydrate content in the feed , lead to higher carbon recovery therefore higher acids productivity.

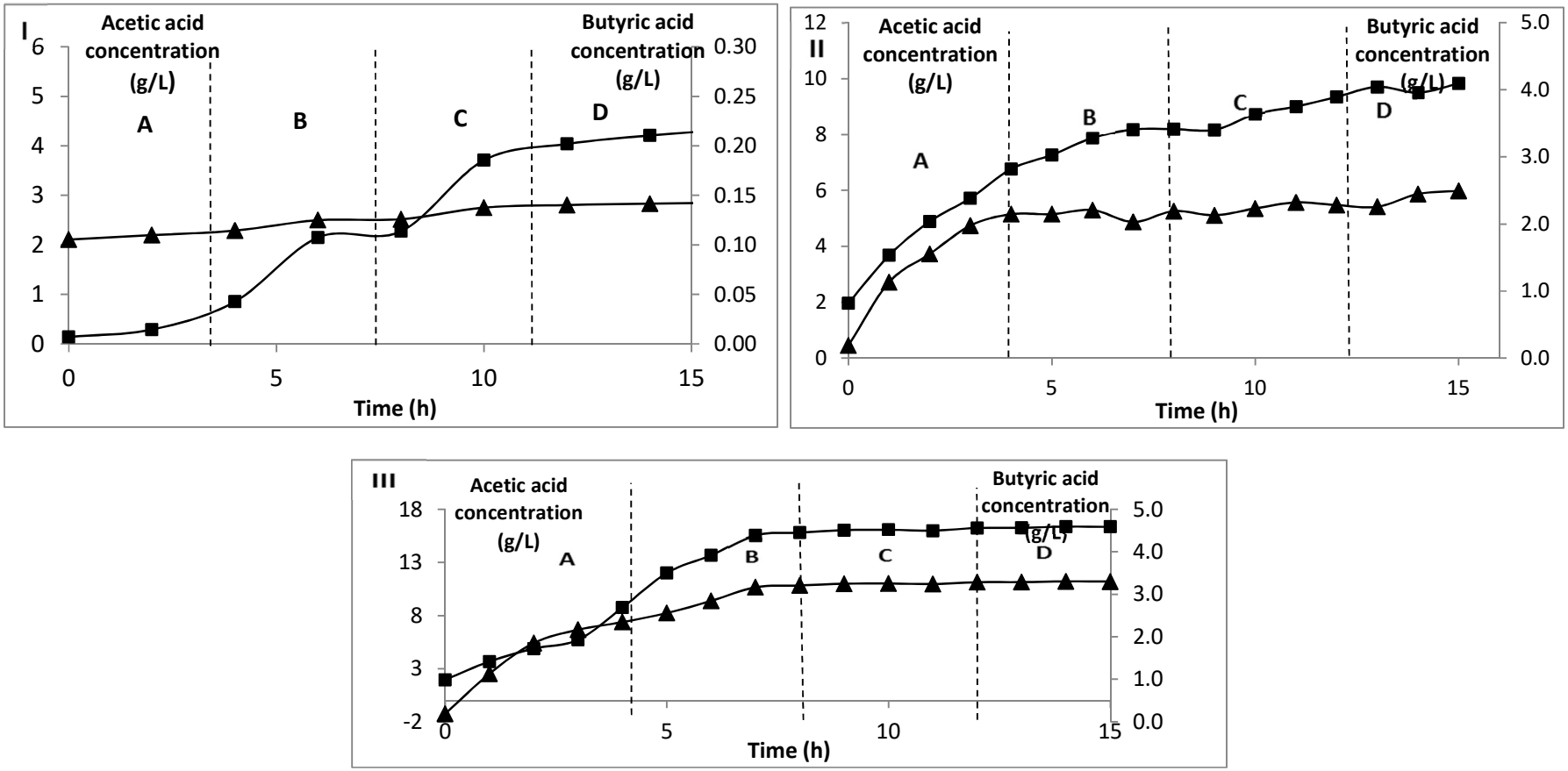

Fig.6 (I, II, III): The production of acetic (■)and butyric acid(▲)by *Cl. butyricum* growth on glucose media at three concentrations of glucose. (I) for 5 g L^{-1} feed,(II) 10 g L^{-1}feed and (III) 15 g L^{-1}, at permeation rates: A: 4 L h^{-1}, B: 8 L h^{-1}, C:16 L h^{-1}, D:32 L h^{-1}

4.5. Cost estimation

The wide usage of this technology, membrane bioreactors for intensive propagation of industrially relevant microorganisms, will depend on its practical and cost effective application to biotechnological production of acids and other bioproducts. Estimation of costs is complex as they arise from a variety of factors, such as energy consumption, addition of water, labour and maintenance and capital costs, such as equipment and scale of operations [45-47]. The value of the products is dependent on the concentration of nutrients present and this may be compared to the costs of equivalent materials such as in vitro nutrient media.

The development of an MBR in an industrial scale (Table 3) has been calculated at £457,416 (Table 4) of which the cost of powdered chemicals for the nutrient media is quite high [48,49]. The calculations are based on 2016 price catalogues provided by nutrient media distributors and manufacturers.

Unit	Element	Type	Surface area (m^2)	Material	Total Cost (GBP,£)	Power Usage (MJ)	Cooling water (kg/d)
MBR	Tank	Processing	37.3	Stainless steel Type 304	74,500	-	17000
		Collection	37.3			-	
	Pumps	Feed	-	Plastic/Metal	2200	37.15	
		Recirculation	-				
	Membrane	Hollow fiber	180	Polymeric (PVDF)	5,680	-	
	Heat Exchanger	Shell and tube	173.2	Stainless steel Type 304	52,000	-	
	Raw materials	Powdered chemicals i.e. yeast extract, glucose, sodium chloride etc. and tap water	-	Powder or liquid	317,034		

Table 3: Major equipment specification and purchase cost (based on 2014) to obtain 220 m^3/d of media[53].

Peer-reviewed version available at Fermentation 2018, 4, 81; doi:10.3390/fermentation4040081

Fixed capital estimate summary	MBR
Total plant direct cost (TPDC) (physical cost)	
Equipment erection	0.4
Piping	0.7
Instrumentation	0.2
Electricals	0.1
Buildings	none required
Utilities	not applicable
Storage	provided in PCE
Site development	not applicable
Ancillary buildings	none required
Design and Engineering	0.3
Variable Costs	
Raw materials	£317,034.00
Miscellaneous materials	£1,316.92
Utilities Cost	
Cooling water	£294.81
Power	£2,227.57
Water	£5500
Shipping & Packaging	not applicable
Fixed Costs	
Maintenance	£13,169.24
Operating labour	£54,000
Plant overheads	£27,000.00
Capital charges	£34,240.02
Insurance	£2,633.85
Local taxes	not applicable
Royalties	none required
Sales expenses	not applicable
General overheads	not applicable
R&D	not applicable
Total annual production rate(rounded)	**£457,416.41**

Table 4: Economic analysis results (based on 2018 prices) to produce carboxylic acids using the MBR system[53,54,61]

However there are potential solutions of subsidising the raw materials with alternative sources. *Cl. butyricum* has the propensity of fermenting multiple carbohydrates, constituting use of vegetative waste such as lignicellulosic hydrolysates possible. These hydrolysates have been advocated as an economic sustainable substrate for the biochemical production of acids. It has though been found that the dilute acid treatment used to generate carbohydrates of them, leads to the formation of microbial growth inhibitors such as phenolics, organic compounds or furan compounds. Depending on various factors such as the origin of lignocellulosic biomass, the usedpre-hydrolysis/ hydrolysis methods, and the applied operational conditions (e.g. pressure, time, pH and temperature) regarding their nature, there are numerous detoxification methods to treat the hydrolysates [62]. Certain *clostiridia spp*. growth and metabolites productivity has been found to be susceptible to the toxic effect of phenolic compounds such as vanillin, furfural and high concentrations of organic acids [62].

Cl. butyricum has been has been grown successfully producing a high amount of carboxylic acids on treated agricultural sludge [33], while other waste sources have been used effectively including industrial wastewaters from food processing industries and breweries [50]. These materials, if used as nutrient media, are potentially highly profitable, especially when compared to the traditional synthetic media or those derived from food sources such as crops. For example, if waste effluents are used instead, the nitrogen sources could be supplemented via organic content in the form of ammonia and the other components such as phosphate and metals. Filtration allows manipulation of the nutrient content, since it can be combined with leaching and acidification using microfiltration or selective separation and concentration using subsequent nanofiltration and reverse osmosis processes. These streams can then be blended enabling the formulation of different concentrations of appropriate proportions suitable for supplying the nutritional needs of microbial fermentations for the intensive production of biofuels, acids and other chemicals.

The productivity of the MBR system will be limited by the permeate rate of the fluid leaving the reactor. Ultimately, the membrane filtration rate will be limited by the cell concentration in the reactor and the consequent cake layer on the filter. Thus, if all other conditions are constant, and the cell concentration increases, then the potential permeation rate will decline. There will therefore be an optimum set of conditions which can be calculated from the maintenance coefficient and the area of the membrane present in the systems.

The use of waste as growth and production fermentation media is effective and economical as well as environmentally advantageous, since the production of powdered yeast extract has a carbon footprint of 0.936 kg CO_2 per kg of material [51,52]. However, even in the case of using powdered materials the cost is potentially offset by the value of the end products. The cost of preparation and production of carboxylic acids using this system was found as 0.0062 £PS/kg respectively.

The MBR is proven a highly beneficial system in terms of cost effectiveness. As biomass is retained in the membrane module, the operational volume is reduced, the streams containing the acids can be further processed by membrane filters, developing an in situ extractive system, eliminating the potential difficulties due to fermentation debris (cellular or medium components) in the recovery of the end products of the microbial metabolism and the toxic effects of the organics acids to the microbial inoculum.

When comparing three systems of culturing the bacterium, batch reactor, chemostat and membrane bioreactor, it is clearly shown that the MBR is a highly productive and efficient system, in terms of carboxylic acids production (Table 5). Previous research has shown that *Cl. butyricum* can grow efficiently in batch reactor, however the system is not as productive. In the chemostat setting, the acetic acid productivity rate was 4.6 times and butyrate productivity rate was 7.7 times more productive than batch system. On the other hand, the MBR system shows 40 times acetic acid productivity rate and 96 times butyric acid productivity rate better than batch system. The productivity of the MBR system will be limited by the permeate rate of the fluid leaving the reactor. Ultimately, the membrane filtration rate will be limited by the cell concentration in the reactor and the consequent cake layer on the filter. Thus, if all other conditions are constant, and the cell

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concentration increases, then the potential permeation rate will decline. There will therefore be an optimum set of conditions which can be calculated from the maintenance coefficient and the area of the membrane present in the systems.

Reactor System	Initial glucose concentration (g L^{-1})	Acetic acid volumetric productivity ($g^{-1}L^{-1}h^{-1}$)	Butyric acid specific cell productivity ($g^{-1}L^{-1}h^{-1}$)	Acetic acid productivity ratio	Butyric acid productivity ratio
Batch	10	0.95	0.15	1.0	1.0
Continuous		4.41	1.27	4.6	8.7
MBR		37.88	14.44	39.9	96.0

Table 5: Comparison of productivity of acetic and butyric acid in the batch, continuous and MBR systems

The main disadvantage of the MBR as an intensive bioprocess based production system of either primary or secondary metabolites is the declining flux due to membrane fouling. Fouling occurs at the interface between the membrane and the mixed liquor. The mechanism is considered rather complicated; however, the main reasons include adsorption of macromolecular and colloidal matter; such as proteins and carbohydrates; growth of biofilms on the membrane surface; precipitation of inorganic matter; and aging of the membrane. Either configuration of MBR's (side stream or submerged) use shear at the membrane surface to prevent fouling. It is likely in the case of side stream MBR's that the shear generated by the pumping will increase cross-flow velocity and thereby supply aeration around the membrane to provide shear stresses. However, the increasing of membrane aeration levels brings an increase in reactor operating costs. This can be solved by new jet and cycling aeration systems if the two systems are employed in membrane bioreactors to control membrane fouling, they are very efficient, and reduce energy demand. Other methodologies have also been suggested to prevent fouling such as aeration of the membrane surface or addition of activated carbon in mixed liquor. Most common method though of fouling prevention remains membrane cleaning. The cleaning protocols are often provided by the membrane manufacturers, while most operators prefer back-flushing technique. Certainly, cleaning methods are crucial to maximise the life expectancy of a membrane bioreactor.

Previous studies [55-57] have been published considering the production of acetic and butyric acid using *C.butyricum*, with varying results, considerably different to the results achieved with this study. In most studies the production of butyric acid is favoured over acetic acid, possibly due to the different substrate composition (molasses, glycerol) and culturing conditions. Interestingly in one study [11] when glucose was used at high concentrations (30 g L^{-1}) the production of butyric acid reached 11.75 g L^{-1} compared to 4.0 g L^{-1} acetic acid.

In this study though, acetic acid production was higher than butyric acid. That can be possibly explained as much of the growth could be derived from yeast extract rather than glucose. In such a case the oxidised constituents can act as electron acceptors, allowing the oxidation of glucose and the formation of acetic acid , different biochemical pathways were used namely the enzymes involved in the integration of carbon and electron flow pathways. An alternate explanation could also be that redox balance must be maintained on glucose, so other end-products would be produced to allow acetic acid to be formed, thus large quantities of hydrogen and other products such as formic acid, glyceraldehyde and possibly glycerol are developed.

In terms of productivity, previous studies were mostly performed in batch setting .The MBR system is much more efficient in terms of carboxylic acid and biomass productivity, since with the system used in this study, 44.89 g L^{-1} and 13.63 g L^{-1} production rates were found for acetic and

butyric acid respectively. Therefore, by the comparison the MBR offers 50 times greater productivity for butyric acid and over 500 times greater for acetic acid when compared to previous studies.

5. Conclusions

Overall, this research has shown the potential using a MBR as a production system for the intensive conversion of carboxylic acids from carbohydrates. The MBR system is most effective compared to other reactor systems although this is technically the most demanding of the three systems studied. Summarising the following points can be

- MBR is the most productive system in terms of cellular biomass and carboxylic acids of the 3 (batch reactor , continuous reactor)
- MBR is a truly beneficial system for intensive microbial culturing due to the ability to work around different modes and culture various microorganisms.
- Depending on culturing conditions/nutrient composition different acids productivities were observed on the 3 systems.
- Depending on culturing conditions/nutrient composition different acids ratios were observed , but overall acetic acid productions was favoured.

Further steps could be taken to constitute the system even more efficient, for example a system of nanofiltration and reverse osmosis membranes could be installed at the product stream line. They could be used to effectively separate the carboxylic acids from the liquid stream and recycle the water back to the reactor, so end product inhibition could be avoided and the acids stream would be concentrated. The high concentration of carboxylic acids produced will be of more value and could be further concentrated or used in other bioprocesses that ferment carboxylic acids to produce polyhydroxyalkanoates (PHAs) and poly-3-hydroxybutyrate (PHB), for example [58-60]. It has been clearly demonstrated that carboxylic acids production can be intensified and potential of such a system to become a viable alternative to conventional anaerobic digestion technology .

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