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Research Article

Production of Acetic Acid and Whey Protein from Cheese Whey in a Hybrid Reactor under Response Surface Optimized Conditions

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Received: 18 January 2023; **Revised:** 2 March 2023; **Accepted:** 10 March 2023

Abstract: Experimental investigations were carried out in a membrane-integrated hybrid bio-reactor system for direct and continuous production of acetic acid and whey protein from waste cheese whey under response surface optimized conditions. In this novel scheme of fermentative production, using *Acetobactor aceti*, the major operating parameters such as temperature, concentrations of yeast extract, and glucose were optimized. Under the set of optimum operating conditions (temperature of 300 °C, pH of 6.0, yeast extract concentration of 15 g/L and glucose concentration of 44 g/L), more than 95% pure acetic acid was produced with a concentration of 84.1 g/L, yield of 97.4% and productivity of 7.2 g/(L· h). Simultaneous production of whey protein powder makes the process more economically viable. Findings indicate that acetic acid could be produced directly and continuously from waste cheese whey with high productivity, yield and purity under response surface optimized conditions, in a simple, flexible, energy-saving and environmentally benign membrane-integrated hybrid reactor system.

***Keywords*:** acetic acid, whey protein, direct production, cheese whey, response surface optimization, membrane, bioreactor

1. Introduction

Cheese-whey is one of the main wastes of dairy industries and sweet meat shops disposal of which in the surrounding environment is not permissible due to high Biological Oxygen Demand (BOD) (30,000-50,000 mg/L) and Chemical Oxygen Demand (COD) (130,000 mg/L).[1,2] This waste contains about 40 g/L to 50 g/L lactose and 60 g/L to 70 g/L protein,[3] which could be used as a raw material fermentation in the production of acetic acid. Pressure-driven (4-6 kg/cm^2) Ultrafiltration (UF) in an appropriate membrane module is used to separate protein and fat contents from cheese-whey exploiting size exclusion principle of ultrafiltration membranes (10-1,000 Å).[4] The ultrafiltration permeate containing whey-lactose can then be subjected to microbial fermentation leading to production of acetic acid. Acetic acid is widely used in production of vinegar, vinyl acid monomer, terephthalic acid, laboratory solvent and many other specialty chemicals.[4] The UF retentate obtained during filtration of cheese-whey is rich in protein[4,5] and can be effectively separated out as a valuable protein source. Simple evaporation of water from whey, in a spray or roll dryer yields whey protein powder. Whey protein containing α-lactoalbumin, β-lactoglobulin, bovine serum albumin

DOI: https://doi.org/10.37256/fce.4120232364

and trace amount of immunoglobulin is widely used for therapeutic purposes, such as production of anti-cancer drugs, and production of whey protein capsules commonly used in body muscle building.[5] Fermentative production of acetic acid from a variety of carbohydrates like glucose, fructose, lactose and sucrose has been reported in the literatures.[6-8] However, the production of acetic acid using renewable raw materials such as cheese whey have been little studied. Moreover, in the reported studies[9-14] acetate salts have been mainly produced through microbial conversion of glucose or equivalent carbohydrate sources by *Clostridium thermoaceticum*, *Acetogenium kivui* and other microbes. Though yield has been reported to be in the range of 90 to 98%, productivity has remained low 0.6-0.7 ($g/L^{-1}\cdot h^{-1}$). Scientists[15] attempted use of waste material in production of acetic acid in fibrous bed bioreactor by the fermentation of sucrose present in corn meal hydrolyzate. In majority of the studies, batch production under controlled pH has been reported using expensive finished carbohydrate sources supplemented with other necessary nutrients. pH control by addition of alkalis almost always invariably leads to production of acetate salts instead of direct acetic acid production.[10-12,16] Efficient separation and purification of the product in the downstream is another big challenge in acetic acid production and several studies.[10,17-22] It was reported on use of micro and ultrafiltration membranes in downstream processing.[23] Though fermentative production has always been carried out under some specific sets of operating conditions, hardly an appropriate optimization technique has been adopted towards arriving at the best conditions for microbial conversion.[17-19] This possibly often resulted in low productivity. The present study is thus an attempt to continuous, direct and fermentative production of acetic acid from a cheap waste material under a properly optimized set of operating conditions in a membrane-integrated hybrid reactor system. Response surface optimization technique is used in arriving at the best operating conditions avoiding the mutual interaction effects of the operating parameters of conventional optimization process. Effort is made to further add economy to the process through recovery of whey protein as a valuable by-product. Such an approach promises a green technology for the production of high-valued organic acid from an industrial waste.

2. Materials and methods

2.1 *Raw material*

Raw cheese whey was filtered by flat sheet cross-flow membrane module using PES-5 membrane with a Molecular Weight Cut Off (MWCO) of 6,000 Da and an operating pressure of 5 kg/cm^2. As a result of its low molecular weight, lactose (0.3 kDa) was permeable through this membrane where proteins were not permeated and was further recycled back to the feed tank as shown in Figure 1. Removal of protein was essential to prevent their inhibitory effect on the specific strain of *Acetobactor aceti*, resulting in a significant decline in product concentration and yield during fermentation. Sweet cheese-whey permeate collected from the permeate side with lactose concentration of 42.4 g/L, was used as the main raw material of carbon source for fermentation.

2.2 *Microorganisms*

Acetic acid producing microbial strain, *Acetobactor aceti* (NCIM 2116) collected from National Chemical Laboratory (NCL, PUNE, India) was used throughout the study. The strain was maintained in both solidified form (on MRS agar slants at 277 K) and in liquid medium. Subcultures were prepared at regular intervals of 30 days and the strain was transferred to fresh medium.

2.3 *Media*

The lactose content (42-43 g/L) of whey permeate was utilized as the carbon source during fermentation. For enhancing microbial growths through reduction of lag phase, the whey permeate was supplemented with glucose and nitrogen rich yeast extracts the optimum doses of which were arrived at through response surface optimization. Salt ingredients $FeSO_4\cdot 7H_2O$ (0.0011 g/L), $CaCl_2$ (0.13 g/L), NaCl (0.8 g/L), K_2HPO_4 (0.5 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4$ (0.2 g/L), $MnSO_4\cdot H_2O$ (0.05 g/L) were added to the media. These were purchased from Sigma Aldrich (USA). Sterilizations were done in glass conical flasks at 121 °C and 15 psi prior to each study.

2.4 *Pilot scale fermentation*

The stirrer speed and temperature were thoroughly maintained at 160 prm and 30 °C. Prior to starting of each experiment, the fermentor was autoclaved at 121 °C and 270 kPa for 15 min and all its tubes were sterilized with ultrapure water at 60 °C till neutralization. The media was inoculated with 10% microbial stock culture as previous. Initially fermentation was carried out up to 48 hours to exploit the exponential growth phase of microbial strain. Whey permeate, which has already been infected and enriched with nutrients, was used directly as the inoculum, which aided the complete elimination of the microbial lag phase of at the beginning of fermentation. Microfiltration was started just from that time (48 h) using flat sheet of Nylon 0.22 membrane in cross-flow module, at a fixed pressure of 1 bar and 0.50 m/s cross flow velocity. Fresh feed (whey permeate with nutrients at pH 6.0) was started to add into the fermentor at the rate of permeate output from microfiltration. The complete schematic diagram of the process has been shown in Figure 1.

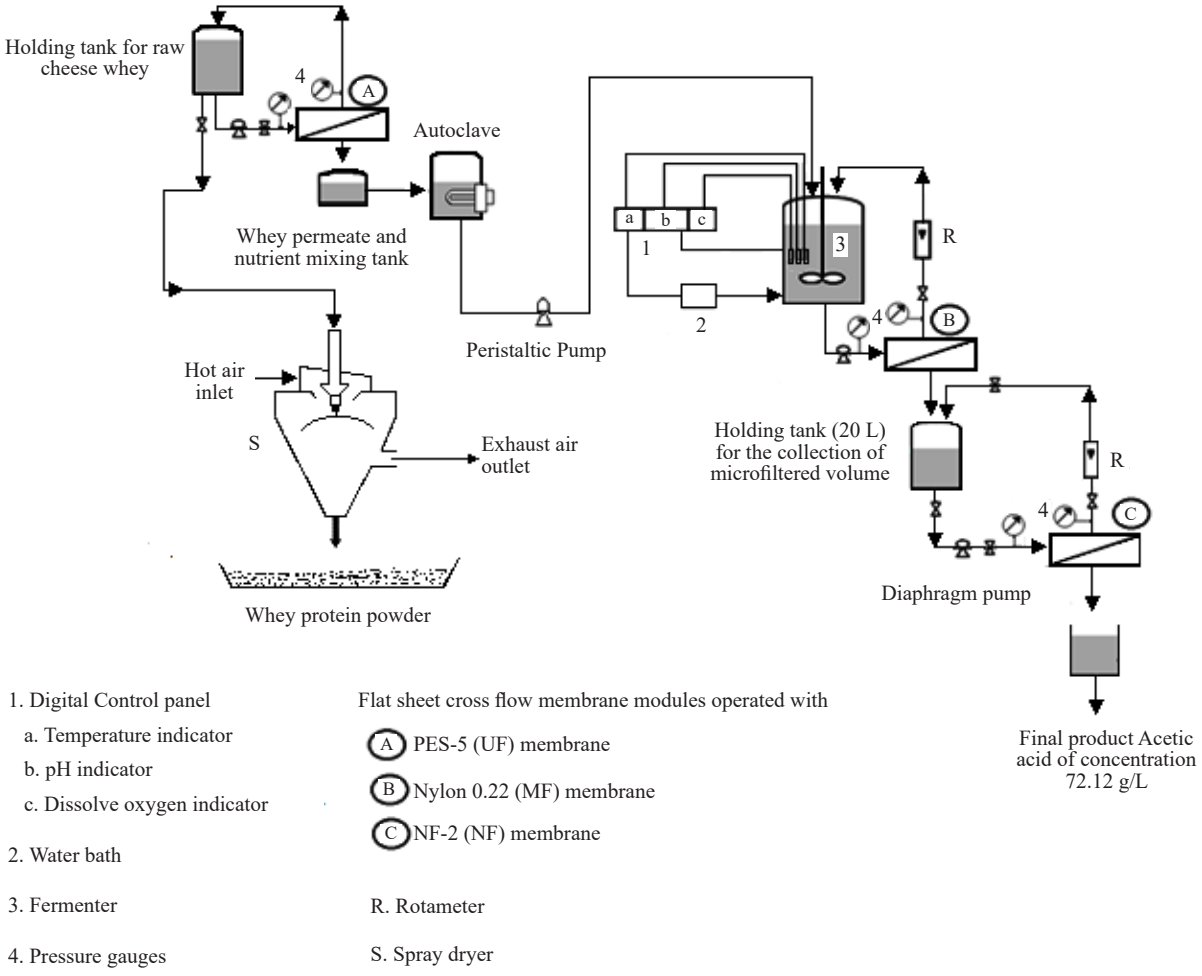

Figure 1. Schematic diagram of membrane integrated system for the production of acetic acid and whey protein

2.5 *Whey protein collection*

The recycle stream from ultrafiltration module was sent back to the cheese whey tank thus increasing the concentration of whey protein in the tank that was subsequently subjected to spray drying to produce whey protein powder. Every 24 hours, fresh cheese whey was inserted into the cheese whey tank. Before that, the 24 hour old protein

concentrated cheese whey solution was sent to the spray dryer at a rate of 2.5 mL/h. Hot air was circulated at a speed of 17.5 m/s inside the spray dryer and whey protein in powder form was recovered from the bottom of spray dryer. In order not to denature whey proteins, warm air circulation was maintained at a temperature below 72 °C.

Table 1. Central composite design of experiments-operational parameters and response

Run	Std.	Glucose concentration (g/L)	YE concentration (g/L)	Temp (°C)	Response: Produced acetic acid concentration (g/L)
1	4	41.89	15.57	25.24	68.23
2	10	50.00	12.00	30.00	84.1
3	5	18.11	8.43	34.76	32.65
4	19	30.00	12.00	30.00	67.65
5	15	30.00	12.00	30.00	67.95
6	18	30.00	12.00	30.00	68.02
7	6	41.89	8.43	34.76	44.05
8	2	41.89	8.43	25.24	47.58
9	13	30.00	12.00	22.00	28.19
10	1	18.11	8.43	25.24	35.06
11	9	10.00	12.00	30.00	48.2
12	20	30.00	12.00	30.00	67.35
13	12	30.00	18.00	30.00	70.9
14	17	30.00	12.00	30.00	67.15
15	3	18.11	15.57	25.24	49.07
16	11	30.00	6.00	30.00	42.68
17	14	30.00	12.00	38.00	23.15
18	7	18.11	15.57	34.76	47.2
19	8	41.89	15.57	34.76	64.56
20	16	30.00	12.00	30.00	67.55

3. Experimental design and optimization using response surface methodology

3.1 *Design of experiments by Design Expert Software*

Supplemented glucose concentration, yeast extract concentration and temperature of fermentation broth were the three significant independent variables, selected as the numeric factors. Using Design Expert Software (version 8.0.4) and Response Surface Methodology (RSM), a total of 20 experiments were created using varying combinations of three process variables, with acetic acid concentration serving as the single response. Number of experiments was determined using the equation $2^k + 2k + 6$, where k denotes the number of independent variables. The factor ranges were input in

terms of α value where the upper (+α) and lower (-α) operational limits of supplemented glucose concentrations were 50 g/L and 10 g/L, respectively. Similarly, the upper and lower limits for the yeast extract concentrations were 18 g/L and 6 g/L, and those for the temperature, were chosen as 38 °C and 22 °C, respectively observed pH was 6.0 at the beginning of fermentation. The experimental conditions designed by RSM, for the optimization of acetic acid concentration along with the respective concentration of the product and the responses have been presented in Table 1.

Fermentation media were prepared according to the different combinations designed by Response Surface Methodology (RSM). Each medium was inoculated with 10% of microbial stock culture and were placed in the incubator shaking at 160 rpm at full throttle for 72 hours. All periodically collected samples were analyzed by High Performance Liquid Chromatography (HPLC) (Agilent 1200 series) for measuring concentration of acetic acid. The model values were fitted and the plot between predicted response and actual response was obtained. 15 different optimized conditions for the operating parameters were produced by RSM. During experimental runs, the conditions leading to negligible difference between the predicted and the actual values were chosen as the optimum ones.

3.2 *Membrane integrated hybrid fermenter system*

Using the optimum values of operating parameters through RSM, continuous mode production of acetic acid in a membrane integrated fermenter of 30 L capacity (made of SS 316) was investigated. This membrane-integrated fermenter unit was fitted with stirrer, and probes for determining pH, temperature and dissolved oxygen. The temperature of the fermentation broth was controlled by using a cold water circulation bath (Metro Tech, India). Downstream processing of the fermentation broth was accomplished with microfiltration, followed by nanofiltration. Flat sheet cross flow membrane modules (each with an effective filtration area 0.012 m^2) were used in all stages of membrane filtration where membrane selection was done in prior studies. Nylon (0.22 μm) microfiltration and NF-2 nanofiltration membranes were found to be the best out of the tested lot in respect of separation factor and flux. Microfiltration was done using Nylon membrane (Membrane Solutions, USA) of pore size 0.22 μm and molecular weight cut off 5,000-100,000 (g/mol). Composite polyamide nanofiltration membranes (NF-2 with average pore size of 0.57 nm) were used in final purification stage. 0.1 N NaOH and 10^{-2} molar HNO_3 solutions in order were used for membrane cleaning followed by back washing after each run. Sterilization of membranes was done using 200 ppm NaOCl solution followed by rinsing with ultrapure water (MILLIPORE Pvt. Ltd, India).

4. Assay procedure

4.1 *Assay of acetic acid and microbial growth*

Cell growth was measured using spectrophotometric analysis. Frequent samples of the fermentation broth were taken and the Optical Density (OD) at 620 nm was measured using a Ultraviolet (UV) spectrophotometer (CECIL, 7000 Series, UK) to determine the stages of microbial growth. Produced acetic acid was analyzed by HPLC (Agilent, Series 1200, USA) along with the measurement of concentrations of residual carbohydrates, namely glucose and lactose. Samples from the fermentation broth were first centrifuged at 10,000 rpm for 15 minutes to ensure the fullest sedimentation of microbial mass and suspended nutrient matters. The supernatants were analyzed to search the acetic acid and residual carbohydrate concentration. Pure acetic acid with a purity level of 99.99% was used to create the standard (Sigma Aldrich, U.S.A.). The concentration of acetic acid was determined using a Diode Array Detector (DAD) coupled to an Ultron ES-OVM Chiral Organic Acid Column (Agilent Technologies). The mobile phase was consisted of acetonitrile (100% pure, Sigma Aldrich, USA) and potassium dihydrogen phosphate (20mili molar aqueous KH_2PO_4 solution of pH -2.0) in a volume ratio of 1:99, at a flow rate 1mL/min with residence time 2.46 min and 10 μL injection volume. Residual carbohydrate (lactose and glucose) concentrations were measured by Refractive Index Detector (RID) equipped with Zorbax Carbohydrate Analysis Column (Agilent, USA). During carbohydrate analysis by HPLC, column temperature was kept fixed at 35 °C where the mobile phase used was 75% acetonitrile and 25% ultrapure water (Milli Q) at a flow rate of 1.4 mL/min where the injection volume of the samples were 10 μL. The Residence Time (RT) values of glucose and lactose were estimated to be 5.32 and 8.8 min, respectively. The purity of the samples after final nanofiltration step was measured using peak purity software tool of HPLC. Specific Electrodes were used to

detect and quantify mineral ions concentrations, such as Na^+, K^+, and Mg^{2+}. (Thermo Scientific, U.S.A.). The mean of the triads was finally accepted in every measurement to smooth out differences and minimise analysis error where the experimental error was computed to be within 1-5%.

4.2 *Assay of whey protein*

Proteins like α-lacto albumin and β-lacto globulin are present in cheese-whey which were identified by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Polyacrylamide Gel Electrophoresis (PAGE) was performed 'stains all' and Coomassie Brilliant Blue (CBB) dyes. A charged molecule in an electric field will move to the electrode that has the opposite charge. The relative mobility of charged species determines how far apart the charged molecules can get. Proteins are separated according to the length of their polypeptide chains using sodium dodecyl sulphate and polyacrylamide, which both effectively remove the effects of the proteins' charge and structure.[24] The concentrations of both these proteins were detected by Lowry's method.[25]

5. Result and discussion

5.1 *Treatment of whey with PES-5, UF membrane with constant cross-flow velocity*

Raw cheese whey was treated with PES-5 Ultrafiltration membrane under constant cross-flow velocity mode. As a result, those particular proteins in retentate stream, concentrated feed tank solution where aqueous solution of lactose was mainly present in permeate stream. The cheese whey sample contained a total of 65 g/L of protein consisting of α-lacto albumin (14.1 KDa) and β-lacto globulin (18.4 KDa). The presence of both the proteins has been identified by SDS-PAGE as in Figure 2.

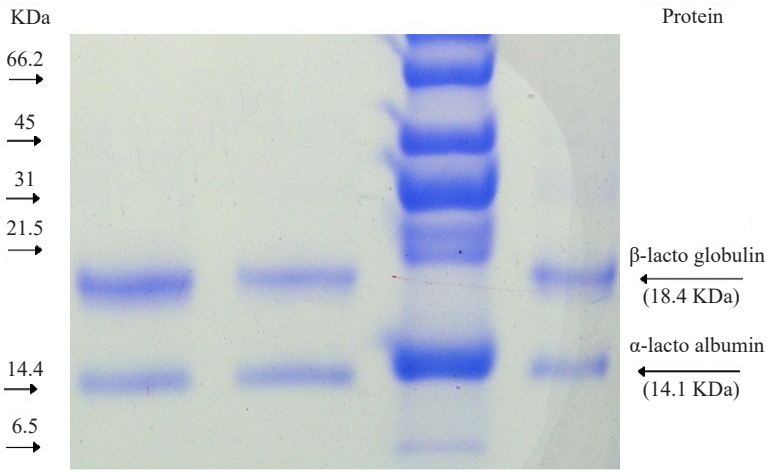

Figure 2. SDS-PAGE for the detection of whey proteins. Visualisation of whey proteins after SDS-PAGE with 'stains all' and coomassie brilliant blue (CBB) dyes. Whey proteins were separated in 16% acrylamide gel and then stained with 'stains all' (A, C) or CBB (B). Lane 1 and 9, protein ladder (10 to 40 µg each protein band); lane 2 and 4, acid whey (55 µg); lane 3 and 5, rennet whey (55 µg); lane 6, β-lg (60 µg); lane 7, GMP (20 µg) lane 8, Mixture of β-lg and GMP (30 µg & 10 µg, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

The permeate flux characteristics with time is represented in Figure 3. As the flat-sheet cross flow membrane modules were put to service, cross flow velocity plays a major role in permeation. At higher cross flow velocity (0.89 m/s) the long-term permeation was facilitated with less extent of pore blocking and was maintained thoroughly at an operating pressure of 5 bar. Lactose was obtained with a concentration of 42.4 g/L in whey permeates which was further converted to acetic acid by fermentative pathway. Similar kind of rejection profile of about 98%-99% to proteins present in casein whey by ultrafiltration membranes was also observed in previous reports.[3]

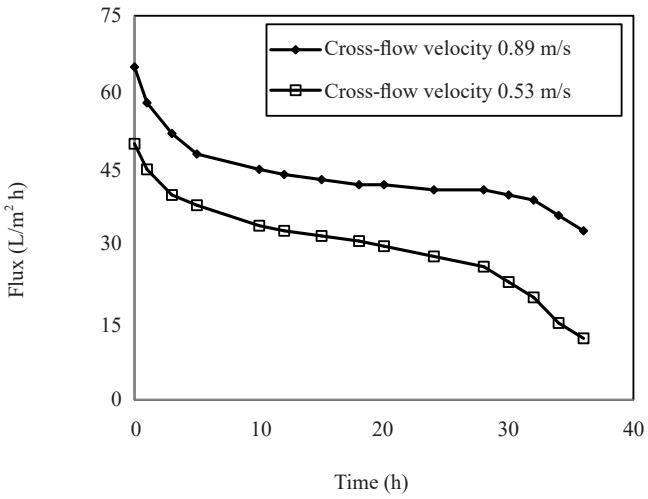

Figure 3. Flux characteristics of ultrafiltration membrane module at constant pressure 4 kg/cm^2 fixed flow rate 0.885 m/s and 0.53 m/s

5.2 *Effect of chosen operating parameters on the fermentation*

Temperature being a major governing parameter was optimized. Among different types of nitrogen rich commercial supplements, yeast extract contains nitrogen including essential amino acids particularly lysine, together with glutamic acid and vitamins[29] which has strong influence rate of microbial growth. Glucose being the simplest carbohydrate could be easily taken up by the microbes resulting in significant enhancement in the cell growth. Thus, the supplemented glucose concentration was optimized by RSM. The combination of the three operating parameters like operating temperature, supplemented glucose and yeast extract concentration exerts influence on acetic acid production. These were required to get optimized avoiding mutual or conjugate interaction effects. The conjugative effects of these three operating parameters on acetic acid production were investigated using Response Surface Methodology while arriving at their optimum values (Table 1). In Central Composite Design (CCD) of the experiments, polynomial of second-order was found to be the best fit while generating the interdependence of the chosen parameters. While performing the analysis of the gathered data, model F-value of 179.98 and the achieved P value (0.0002) being much less than 0.0500 indicated that the model was significant. Through analysis of variance (ANOVA), an empirical relationship of the concentration of acetic acid with the three operating parameters was established. The significance of each term in the equations and how well they fit in the equation could be estimated by this analysis.[26] The obtained empirical equation in terms of coded factors (generated by RSM) may be expressed by the following equation:

$$\text{Acetic acid concentration (g/L)} = +67.60 + 8.85 \times A + 8.58 \times B - 1.46 \times C + 1.58 \times A \times B - 0.36 \times A \times C + 0.050 \times B \times C - 0.49 \times A^2 - 3.80 \times B^2 - 14.808\ C^2$$

A, B and C represent supplemented glucose concentration (g/L), supplemented yeast extract concentration (g/L); and temperature (°C) respectively. The value of the adjusted regression coefficient (0.9883) was within reasonable agreement with the predicted regression coefficient (0.9540), which also implied that the model was significant. The generated curve between the experimentally obtained response value and the model predicted values of acetic acid production has been shown in Figure 4, where the points are present in a clustered fashion around the diagonal line. This explains the high significance of the model along with its capability to predict the experimental values of response.

The combined effect of temperature, concentration of glucose and the yeast extract supplement was analyzed in the ANOVA section where the response curves were found a surface plots. The coupled effect of temperature and yeast extract on the acetic acid production has been described in Figure 5. The figure indicates that the concentration of produced acetic acid increased with the increase of temperature up to 30 °C, but beyond this temperature, acetic acid concentration decreased with further rise of temperature. During the fermentation at operating temperatures below 30

°C, acetic acid concentration was quite low despite high concentration of the yeast extract.

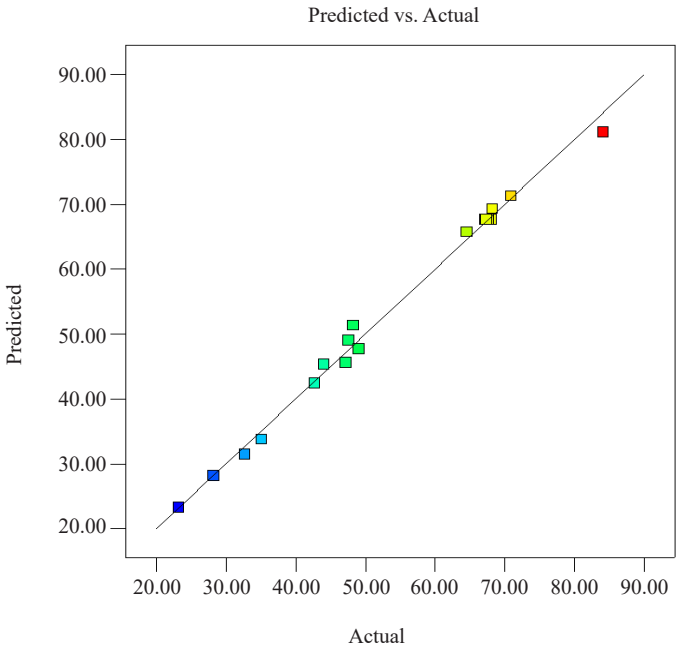

Figure 4. Distribution of model predicted values versus experimentally obtained values of response

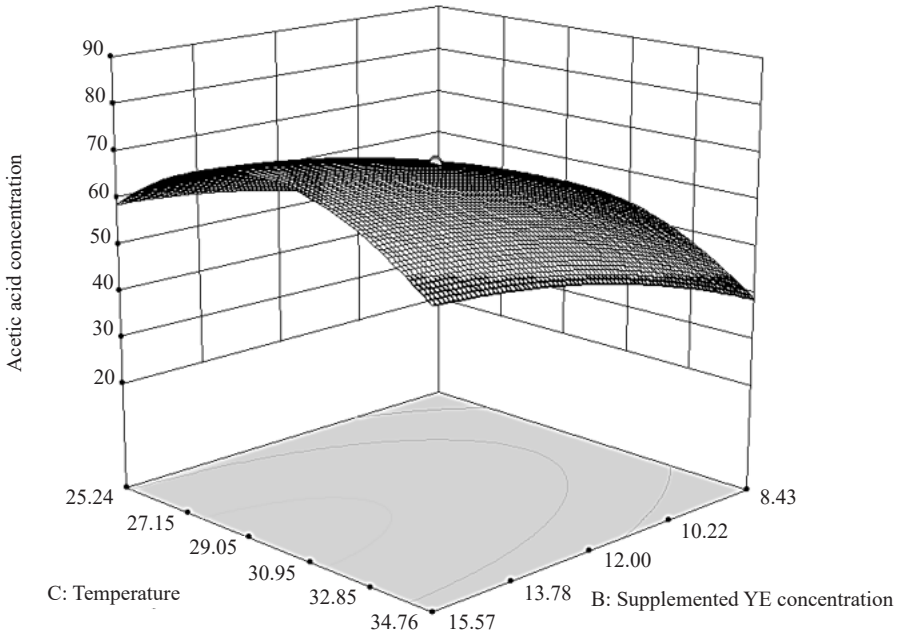

Figure 5. Response surface plot describing the effect of temperature and supplemented yeast extract concentration on acetic acid production

The surface plot in Figure 6 describes variation of acetic acid concentration with temperature and supplemented glucose concentration which shows that, at low temperatures (near 25 °C), glucose concentration did not have any significant effect on acetic acid production. Elevation of temperature close to 30 °C, along with higher glucose

concentration generated a positive effect on acetic acid production. Concentration of yeast extract beyond a certain level (15 g/L) did not exhibit significant improvement in acetic acid production.

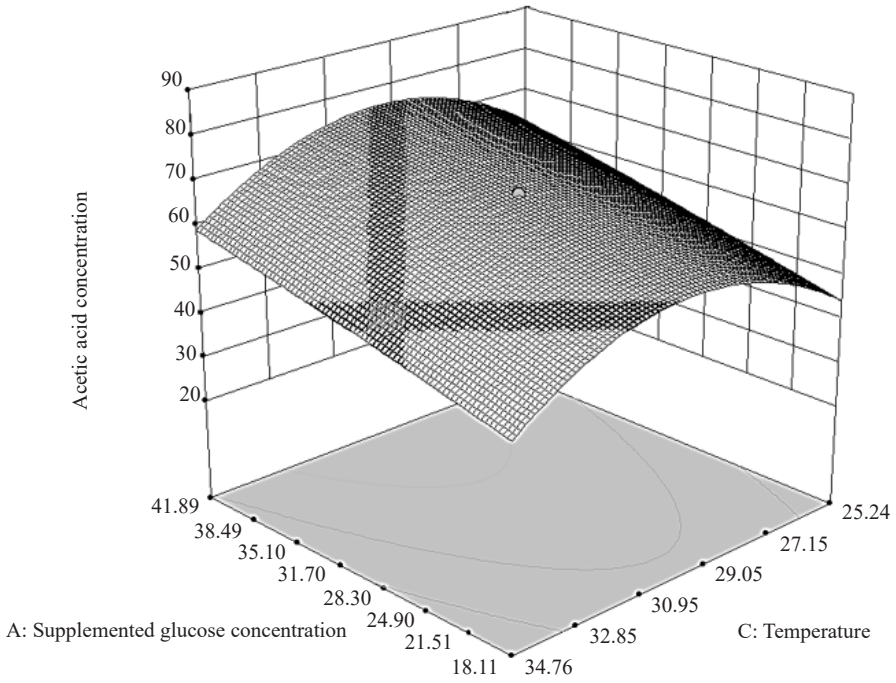

Figure 6. Effect of temperature and supplemented glucose concentration on acetic acid production

At the final stage of optimization, the selected criteria for temperature (A), yeast extract (B), and peptone concentration (C) was within desired range and acetic acid concentration (R1) was at the maximum level. From the suggested optimized solutions with different values of operating parameters, performed by the software, the optimum set of solution with the highest desirability (0.994) consisted of pH value of 6.0, temperature of 30 °C, yeast extract concentration of 15 g/L and glucose concentration of 44 g/L when lactic acid concentration was 83.42 g/L. The achieved final concentration of acetic acid after RSM was 83.86 ± 0.83 g/L with an average product yield of 97.1%.

5.3 *Substrate inhibition*

Production of acetic acid following fermentative pathway is a process purely associated with microbial growth where to some extent, acetic acid also occurs without any growth of microbial growth. This ultimately leads the process to follow Luedeking-Piret model which indicates that the production is not based only on the microbial growth. Additional simple carbohydrate (glucose) was added to the fermentation media with a viewpoint to enhance microbial growth and desired product. Therefore, it was expected that at the highest level of glucose, the product concentration should reach at some higher value. However, this idea does not hold for standard 10 run 2 (Table 1) where understandably the model predicted value is fairly deviated from the experimentally obtained value. This drew our concern to survey the effect of substrate concentration with an aim to examine the occurrence of substrate inhibition. Investigation for the effect of mixed substrate concentrations (glucose and lactose) in batch cultures for the acetic acid production process turned necessary as high substrate concentration may produce an inhibitory effect on the cell growth while blocking the active sites of the enzymes.[27] The active sites of enzymes, present in the microbial body are responsible to carry out fermentation leading to the formation of the desired product. As the concentration of whey lactose in cheese whey is constant, so some fresh experiments were carried out by varying the supplemented glucose concentration within the range of 30-50 g/L while the optimized other two parameters (temperature and yeast extract

concentration) were maintained at their optimum values. The media (1 to 4) were prepared with different glucose supplementation such as 30 g/L, 40 g/L, 44 g/L and 50 g/L.

The fermentation was run for long 96 hours from where arises a question of a proper operating time. According to Figure 7, up to 52 hours of fermentation period, microbes were in fully exponential growth phase, after which there occurred the onset of stationary phase.

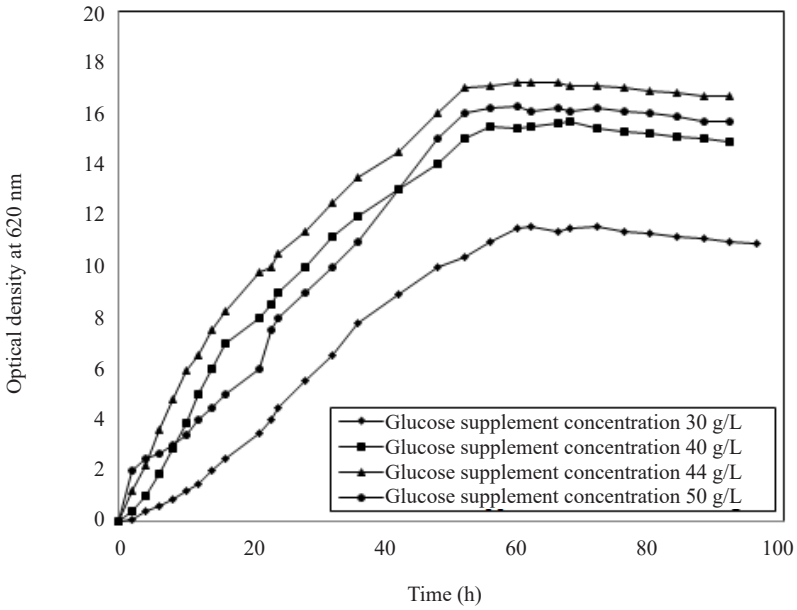

Figure 7. Microbial growth profile in terms of optical density; experimental conditions: pH = 6.0, Temp = 30 °C, Yeast Extract concentration = 15 g/L, glucose supplement concentration 30-50 g/L in cheese whey permeate

However, due to non-growth associated product generation, acetic acid production was active for some hours (10 hours). After 62 hours, the concentration of acetic acid in the fermentation broth turned constant for all glucose concentrations. The generation of acetic acid and the consumption of carbohydrate during fermentation broth have been represented in Figure 8. Further fermentation did not facilitate further generation of acetic acid inside the fermentation broth. Achieved results perfectly support the results as reported,[28] where the growth rate and acetic acid production using *Acetobacter aceti* IFO 3281 was investigated for a long period of 192 hours. The highest concentration of acetic acid produced was 83.86 g/L, with a peak yield of 97.1% and productivity of 1.35 g/(L·h) using a glucose concentration of 44 g/L after 62 hours of fermentation. After the completion of 96 hours of fermentation time, there was no significant improvement of substrate to product yield but with a significant reduction in productivity from 0.87 g/(L·h) to 0.44 g/(L·h) was observed.[28] As a conclusion, the operating time for this system should be 62 hours. Moreover, the supplementation of glucose at concentrations greater than 44 g/L along with whey lactose could not produce any more significant effect on the acetic acid generation. The addition of glucose at 50 g/L resulted in an acetic acid production of 84 g/L after 88 hours of fermentation with a resulting yield of 90% and productivity of 0.95 g/(L·h).

This result suggests that the best glucose concentration to use in fermentation process to produce high level of acetic acid is 44 g/L. Use of glucose supplement concentration of 50 g/L, resulted the product concentration of 84 g/L after 88 hour of fermentation with the resultant yield of 90% and productivity of 0.95 g/(L·h) suggests glucose concentration of 44 g/L as the best one. The optimized operating conditions along with the produced concentration of acetic acid have been represented in Table 2.

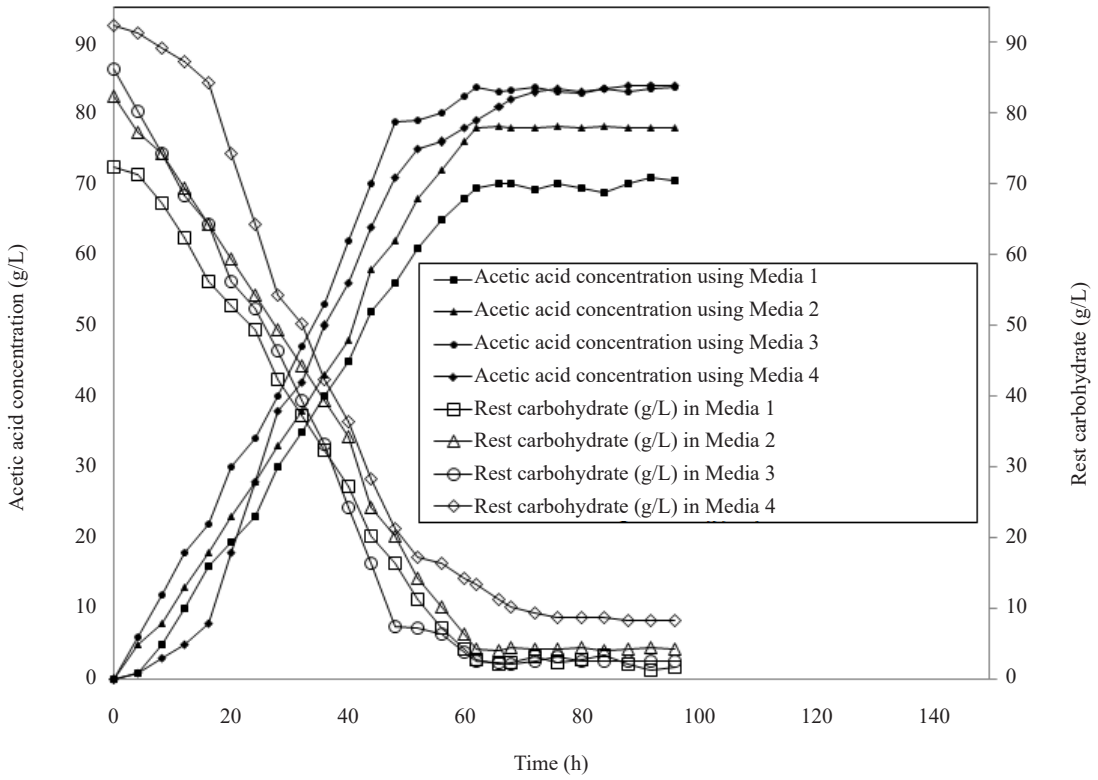

Figure 8. Acetic acid production at different concentrations of glucose supplement

Table 2. Performance of a fermentor under response surface optimized conditions at pH 6.0

Optimum supplemented glucose concentration (g/L)	Optimum temperature of operation (°C)	Optimum supplemented yeast extract concentration (g/L)	Optimum time for fermentation (g/L)	Concentration of acetic acid (g/L)	Yield (%)	Productivity (g/L·h)
44	30	15	62	83.86	97.1	1.35

5.4 *Pilot scale fermentation maintaining a fixed dilution rate*

After observing the effects of operating parameters in shaker flask, the values of those parameters were implemented and maintained while working in pilot scale for the direct production of acetic acid. The microbial growth (in terms of optical density), production of acetic acid and consumption of lactose with respect to time has been represented in Figure 9.

The results show an increase in microbial volume with a substantial increase in the production of acetic acid in the fermenter immediately after microfiltration. After reaching of steady state, the produced acetic acid concentration slowly got diminished and followed a constant trend with respect to time. The steady state condition was achieved at the 5th hour of beginning of microfiltration (Figure 10). At steady state, the microfiltration permeate output was observed to be 66 L/(m^{2}h) maintaining a dilution rate of 0.1 h^{-1}. Survey for the effect of dilution rate was carried out from 0.1 h^{-1} to 0.4 h^{-1}. It was observed that fermentation at the lowest dilution rate (0.1 h^{-1}) resulted in the highest concentration of produced acetic acid (84.1 ± 0.6 g/L) with 97.4% product yield. Although in case of higher dilutions, higher productivity was achieved but the maximum conversion of carbohydrates to acetic acid was unachievable resulting low yield.

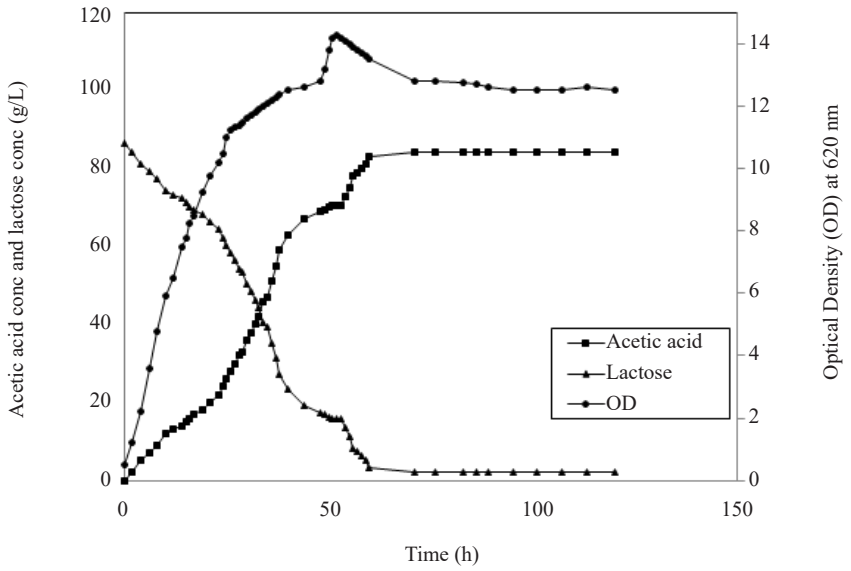

Figure 9. Continuous production of acetic acid under optimum conditions: pH = 6.0, Temp = 30 °C,Yeast Extract concentration = 15 g/L, glucose supplement concentration 44 g/L at Cross flow velocity = 0.53 m/s during microfiltration while maintaining a dilution rate of 0.1 h^{-1}

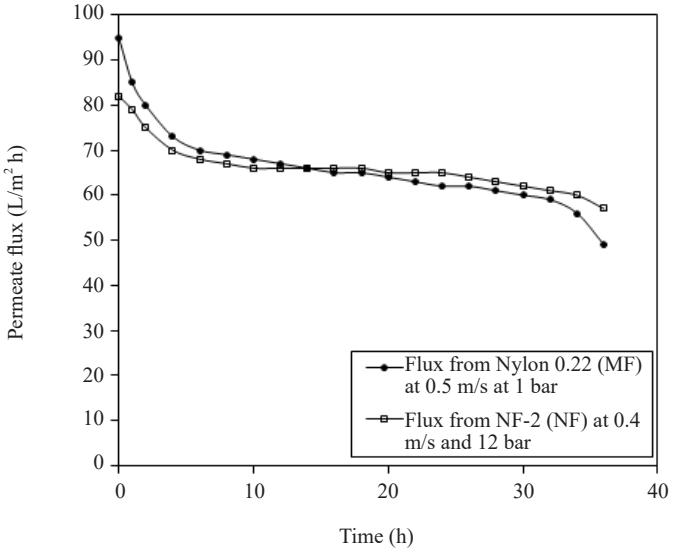

Figure 10. Flux profile with 3 active Nylon 0.22 microfiltration modules and one NF-2 nanofiltration membrane module

Moreover, with the increase of dilution rates the productivity slightly increases but produced acetic acid yield greatly decreased. pH of the fermentation broth was 6.0 at the beginning of fermentation which later on reaches a constant value of 3.2 due to continuous separation of acetic acid using highly selective membranes. The microfiltration process was adapted as a primary treatment technique to satisfy the aim to separate microbial bodies from the fermentation broth.

5.5 *Downstream processing of fermentation broth*

The nutrients like yeast extract and other salts present in the fermentation broth increases the impurity level

which should be separated with efficient and effective downstream processing units. Traditional acetic acid production processes use the heat treatment mechanisms like distillation, evaporation and crystallization which consumes high energy cost for the production of acetic acid. Utilization of membranes with high selectivity, stands to be a highly efficient technology with low energy consumption as it involves no phase change. Membrane units being modular in design, provides the opportunity to turn the plant into a flexible one while ensuring high process intensification. Three microfiltration units followed by a single nanofiltration membrane module exploiting NF-2 membrane were used for downstream processing. For the circulation of broth, a peristaltic pump (Entertech, India) was integrated. As the microfiltration permeate flux reached steady state, the first nanofiltration process was started where the operating pressure and flow rate were so maintained that could generate the identical flux as in the case of microfiltration. The flux characteristics of NF-2 is represented in Figure 10. At 6th hour of the starting of Nanofiltration, the steady state permeate flux was attained. This crossflow nanofiltration process was run with only a single module using a high-pressure HYDRA-CELL diaphragm pump (USA), exploiting NF-2 membrane (pore size 0.53 nm) at an operating transmembrane pressure of 12 bar and cross flow velocity of 0.4 m/s. Thus the rate of permeate output from microfiltration system was equal to the rate of permeate output from nanofiltration system which turned equal to the fresh feed input rate to the fermentor. No significant flux decline or fouling was observed in nanofiltration as the fermentation broth was previously treated by microfiltration membrane system.

The generated clear, transparent aqueous solution of acetic acid post to nanofiltration treatment was 95.2% pure. The concentration of pure acetic acid after nanofiltration turned 72.12 g/L because acetic acid suffered 14% rejection at 12 bar (Figure 11) by NF-2 membranes due to which the overall productivity for the overall pilot plant system turned 7.2 g/(L·h).

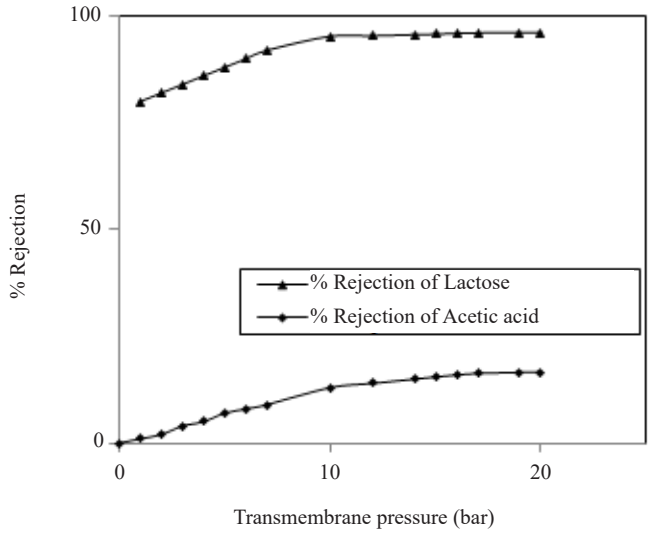

Figure 11. Rejection characteristics as shown by the NF-2 nanofiltration membrane

Current research conducted for this study leads to the conclusion that fermentation processes for producing acetic acid can be significantly enhanced through the optimum adjustment of operational parameters using response surface methodology by studying the combined effect on the concentration of produced acetic acid. Individual and conjugated effects of temperature, supplemented glucose and yeast extract concentrations were professionally investigated in this study. The optimization of the operating parameters and their application during the lactose fermentation has generated an optimal production of highly concentrated pure acetic acid. The optimum temperature thus found out was 30 °C. Finally, the maximum acetic acid concentration of 84.1 g/L with maximum product yield of 97.4 % and overall productivity of 7.2 g/(L·h) was achieved from cheese whey permeate at pH 6.0, using optimum values of yeast extract of 15 g/L and glucose of 44.0 g/L. Yeast extract turned out to be the most significant nutritional parameter, due to the

variation of which, production gets largely affected. Adjustment of pH at 6.0 before the starting of fermentation in membrane integrated fermentor, turned out to be a microbial growth enhancing factor. Productivity, which is basically the output from a reactor per unit time, was improvised remarkably by using response surface optimization technique. Thus, the pollutant whey was renewed to acetic acid which is highly valuable in modern life with an outstanding result as compared to the previous literatures.[16-24] Previous studies mostly encircle on acetic acid production but, not on the recovery of such high valued product like whey protein from the by-product stream. After reviewing all the previous works, it was observed that though the obtained yield of acetic acid is in reasonable agreement with those of the previous works, the obtained productivity which is basically the output from the reactor per unit time, stands to be the highest amongst all the cases.

As an overall, this production technology describes the production of highly concentrated acetic acid with high yield, productivity and purity under a non-neutralizing fermentation-based process after proper optimization of thermal and nutritional factors from cheese whey, a cheap and renewable carbon source. Thus, this scheme for acetic acid production from such renewable substrates under response surface optimized conditions could show a very promising economical pathway of production and to meet up the global demand.

5.6 *Recovery of by-product: whey protein*

The cheese whey contained around 65 g/L protein. Due to continuous extraction of whey permeate and continuous recycling of the protein portion from the cheese whey holding tank, the remaining volume of cheese whey got concentrated. After a long 24 hours of protein recycling by ultrafiltration, the protein concentration in the holding tank reached a level of 480 g/L. The concentrated solution was sent to the spray dryer and whey protein was recovered in the form of fine powder.

6. Conclusions

The main advantage of this process is that the raw material for carbon sources may be obtained at a negligible cost. By proper optimization of the operating parameters using response surface methodology, production of acetic acid in terms of productivity, yield and product concentration were substantially improved. Use of highly selective microfiltration and nanofiltration membrane modules helped production of highly pure acetic acid. Continuous removal of produced acid did not allow pH value to go down beyond the desired level. The modular design of the membrane system offers flexibility in production volume. This no-phase change process (barring protein drying) is basically an energy-saving one. Recovery of whey protein improved economy. This novel scheme permits direct production of highly pure and concentrated acetic acid and whey protein from dairy wastewater.

Acknowledgement

Authors are sincerely thankful to Department of Science and Technology (DST-FIST program) of the Government of India for the financial support. The authors acknowledge thanks to National Chemical Laboratory, Pune, India, for providing the necessary microbial strains.

Conflict of interest

Authors declare no conflict of interest.

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