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Production of lactobionic acid with process integrated electrochemical enzyme regeneration and optimisation of process variables using response surface methods (RSM)

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Abstract

This study focussed on the continuous electrochemical regeneration of cellobiose dehydrogenase, which has excellent biocatalytic properties. Conversion of lactose to latobionic acid was used to measure catalytic activity. The results obtained were compared with the results for the enzymatic regeneration method. Moreover, medium components were optimised using response surface methods for the turnover frequency of the biocatalyst. No loss of activity for the biocatalyst was observed due to contact with electrodes. Overall, this electrochemical process promises to be easier to control, more cost-effective and environmentally friendly than enzymatic processes. The product lactobionic acid can be widely applied because of its non-toxic and biodegradable character.

Keywords: Lactobionic acid; Electrochemical regeneration; ABTS; CDH; RSM

1. Introduction

Cellobiose dehydrogenase (CDH, EC 1.1.99.18), a hemoflavoprotein, is an extracellular enzyme produced by wood-degrading fungi. It oxidises various substrates such as lactose or soluble cellodextrins and mannodextrins to the corresponding lactones, via a ping–pong mechanism using many electron acceptors including quinones, phenoxyredicals, Fe^{3+}, Cu^{2+} and tri-iodide ion [1]. One major obstacle for the use of CDH as a biocatalyst is the regeneration of the active enzyme species, which showed negligible activity during regeneration by oxygen. Furthermore, oxygen is reduced to hydrogen peroxide, which is detrimental to enzyme stability [2].

The enzyme can transfer electrons to various acceptors such as cytochrome *c*, 2,6-dichloro-indophenol (DCIP), 1,4-benzoquinone, 2-2′-azinobis-(3-ehtylbenzothiazoline-6-sulfonate) (ABTS) and metal ions, etc. [3]. Consequently, a combination of an electron acceptor (ABTS) and a regenerating enzyme (laccase) was studied [2]. In this case, the reduced CDH, resulting from the oxidation of lactose to lactobionic acid, was re-oxidised by the mediator, thus reducing laccase and converting the electrons to molecular oxygen (Fig. 1).

The use of a laccase leads to a rise in production costs (because of the additional enzyme) and creates additional limitations for the overall reaction. The pH chosen must be an optimum value for both enzymes (CDH and laccase). Since mediator oxidation should not be a limiting factor, concentrations have to be high enough for both the mediator (ABTS) and the regenerating enzyme (laccase). This leads to low ‘total turnover numbers, ttns’, calculated as moles of product formed per mole of catalyst/mediator used. However, the most severe limiting factor is the oxygen transfer to the solution, which limits the overall reaction rate [2]. To combat this, anodic oxidation of the redox mediator was used for enzyme regeneration in this study (Fig. 2).

This new system has the advantage of operating at the optimum pH value for CDH activity and of controlling mediator concentration [4], thus overcoming the restrictions to regeneration. Moreover, by eliminating the second enzyme from the reaction sequence and by using electricity instead of molecular oxygen, the overall process will become more cost-effective

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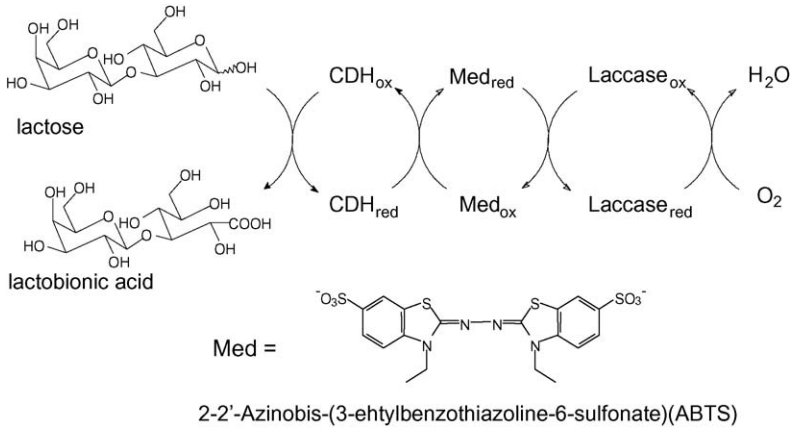

Fig. 1. Enzymatic regeneration of CDH using ABTS, laccase and molecular oxygen as reported by Baminger et al.

and environmentally friendly [5]. ABTS has been successfully applied for cofactor-dependent dehydrogenase and investigated for the racemic resolution of 1,2-diols [6].

Lactobionic acid, formed by oxidation of disaccharide lactose (milk sugar), is currently being used in the pharmaceutical industry as a salt form for intravenously delivered erythromycin [7] and in mineral supplementation [8]. However, its greatest commercial use is as a major constituent of organ preservation fluids during transplantation procedures [9]. One more promising application is the use of lactobionic acid as a cobuilder substance in European detergents as it is biodegradable and non-toxic [10].

This study aimed at developing an electrochemical process for the oxidation of lactose to lactobionic acid using CDH as well as optimising the medium components, the substrate (lactose), the redox mediator (ABTS) and the electrolyte for maximum 'turnover frequency' (***tf***) of the biocatalyst (CDH), calculated as moles of substrate catalyzed (or moles of product formed) per mole of biocatalyst used per unit time.

The traditional one-factor-at-a-time approach to optimisation is time-consuming and incapable of reaching a true optimum because it does not consider interaction among factors. In contrast, statistical methods can take into account the interaction of variables in generating the process response. Response surface methods (RSM) consist of a group of empirical techniques devoted to the evolution of relations existing between a cluster of controlled experimental factors and the measured responses, useful for developing, improving and optimising processes by carrying out a limited number of experiments. In this study, 'central composite design', was applied.

2. Experimental

2.1. Chemicals

All the chemicals used were of analytical grade (Sigma–Aldrich) and were not purified further. CDH and laccase were from white mould and *Trametes versicolor*, respectively.

2.2. Enzyme activity tests

CDH activity was tested by following the decrease in the absorbance of the ABTS radical cation (produced separately in an electrolysis cell) at 414 nm ($\varepsilon_{414} = 31{,}100\ \mathrm{mol^{-1}\ cm^{-1}}$). For preliminary tests the reaction mixture contained lactose (30 mM) and the ABTS radical cation (0.6 mM) in a 20 mM Na-acetate buffer, pH 4.0. At a later stage, the buffer composition was changed (Section 3.2). Stoichiometrically, one mole of CDH, when oxidised, reduces two moles of the ABTS radical cation. Hence in this case, one unit activity of enzyme was defined as the amount of enzyme required to reduce 2 μmol of ABTS radical cation in 1 min at room temperature. For laccase, one unit activity was defined as the amount of enzyme oxidizing 1 μmol of ABTS per minute at room temperature.

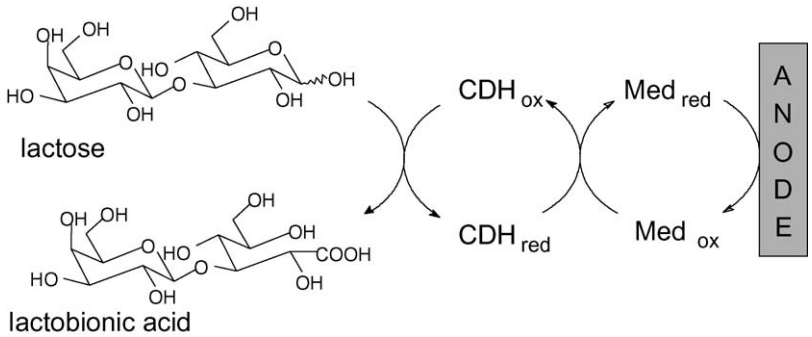

Fig. 2. Electrochemical regeneration of CDH via anodic oxidation of the redox-mediator: ABTS.

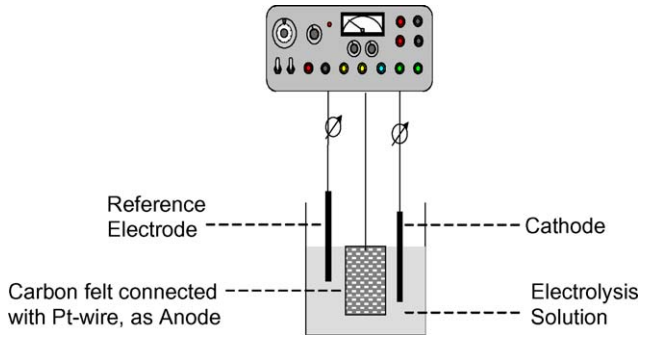

Fig. 3. Electrolysis cell set-up.

2.3. Electrochemical set-up

The electrolysis cell (Fig. 3) was set-up over a magnetic stirrer in a 25 ml volume laboratory beaker. Carbon felt (4.34 cm^2, 0.106 g) connected with Pt-wire (Ø0.25 mm) acted as an anode, Pt-wire (Ø0.5 mm) as a cathode and Ag-wire (Ø1.0 mm) as a pseudo-reference electrode. Later, Ag/AgCl reference electrode was available (MF 2052, BASi, UK). Constant potential difference (500 mV) was maintained between the cathode and the anode using a mini potentiostat (Bank Elektronik, Germany). Continuous stirring induced homogenisation within the reaction volume.

2.4. Experimental design and optimisation by RSM

The range and the levels of the experimental variables under study are shown in Table 1. The investigation was carried out at a fixed CDH concentration (0.1 U/ml) in the reaction mixture. The objective was to determine the optimum concentrations of lactose and ABTS for maximum turnover frequency for CDH. Based on enzyme kinetic studies, the central values (zero level) chosen for the experimental design were 60 and 0.1 mM for lactose and ABTS, respectively.

A central composite design [11] was chosen, consisting of four runs at the corners of a square $\{(x,y) = (-1,-1), (1,-1), (-1,1), (1,1)\}$, plus four runs at the centre $\{(x,y) = (0,0)\}$, plus four axial runs $\{(x,y) = (-1.414,0), (1.414,0), (0,1.414), (0,-1.414)\}$. The data obtained from these experimental runs was used to develop the second order regression equation:

$$Z = a + bX + cY + dX^2 + eY^2 + fXY + \varepsilon$$

where Z is the measured response, the ***tf*** (h^{-1}) and X and Y are variables as values for millimolar concentrations of lactose and ABTS respectively. a is the intercept term, b and c measure the linear effects while d and e account for the quadratic effects of the variables. f represents first-order interaction between X and Y. ε is the error term.

'TableCurve 3D' software (Version 4.0, SYSTAT Software Inc.) was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables were obtained by analysing the response surface contour plot and solving the regression equation.

Table 1
Central composite design consisting of 12 experiments for the study of two experimental factors: in coded values (x and y) and the respective millimolar concentrations

Run no.	x	Lactose (mM)	y	ABTS (mM)
1	0	60	0	0.1
2	0	60	0	0.1
3	0	60	0	0.1
4	0	60	0	0.1
5	−1	45	−1	0.05
6	−1	45	+1	0.15
7	+1	75	+1	0.15
8	+1	75	−1	0.05
9	0	60	−1.414	0.0293
10	−1.414	38.79	0	0.1
11	0	60	+1.414	0.1701
12	+1.414	81.21	0	0.1

2.5. Analytical methods

Lactose and lactobionic acid were analysed by HPLC using a NUCLEOGEL ION 300 OA (300 mm × 7.7 mm; Macherey-Nagel, Germany) at 70 °C with milliQ-water as an eluent (0.4 ml/min) and Electron Light Scattering Detection (SEDEX 75, SEDERE, France). Prior to analysis, samples were filtered using VIVASPIN 0.5 ml CONCENTRATOR (Saartorius, Germany). For enzyme activity tests, the decrease in absorbance for the ABTS radical cation was determined at 414 nm using UV 2102 PC Spectrophotometer (Shimadzu, Japan).

3. Results and discussion

3.1. Preliminary electrolysis

Based on literature [2], the regeneration and catalytic activity of CDH were measured in terms of the amount of lactose being oxidised to lactobionic acid per unit time. For comparative purposes, two sets of experiments were conducted simultaneously (50 mM lactose, 20 mM Na-acetate buffer, pH 4.0, RT), in which the redox mediator and in turn the biocatalyst (CDH) were regenerated by electricity and laccase (further regenerated by molecular oxygen), respectively. The enzymatic cell was oxygenated by sparging the solution with pure oxygen (0.3, v/v min^{-1}). As the amount of CDH available was very limited, the working concentrations of the catalyst were kept low (0.1 U/ml CDH) and concentrations of laccase (0.36 U/ml) as well as ABTS (0.11 mM) were reduced at the same ratio as in the enzymatic process [2]. These first results showed that via electrochemical regeneration, it was possible to reach quantitative conversions not achieved by the enzymatic regeneration system under similar conditions (figure or data not shown). No loss of enzyme activity was observed due to contact with electrodes. In contrast to enzymatic regeneration, there is no limitation to the availability of the final electron acceptor in the electrochemical regeneration process. Moreover, monitoring is simple, since only the current flow in the electrolysis cell at a fixed potential is monitored.

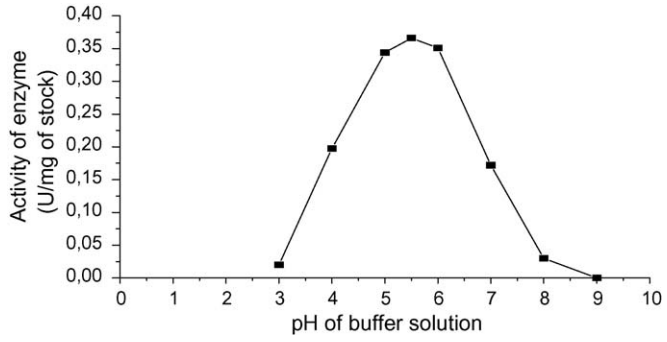

Fig. 4. Activity of CDH at varying pH values of the buffer solution.

3.2. Screening for suitable buffer

For the preliminary experiments, the buffer was kept the same as in previous studies [2,3]. As was already reported [12] the pH of the electrolyte alters the internal electron transfer in the CDH, hence its catalytic activity. The first objective was to define an optimum buffer for CDH activity. From previous experience with electrolysis [4] the concentration of the buffer was defined at 100 mM. To determine a suitable pH, the following buffers were tested (Fig. 4):

- For pH value between 3.0 and 5.5: Na-acetate buffer (pK_a 4.76);
- For pH value between 6.0 and 9.0: Na-phosphate buffer (pK_a 7.2).

The results were compared in terms of activity obtained per milligram of enzyme stock (one unit activity of the enzyme was defined as the amount of enzyme required to reduce 2 μmol of ABTS radical cation in 1 min).

Since the biocatalyst showed maximum activity in Na-acetate buffer at pH 5.5, this value was used for all the following electrolysis processes.

3.3. Enzyme kinetic assays

The assays were carried out in 100 mM Na-acetate buffer at pH 5.5 for both lactose and the ABTS radical cation (Table 2), following the steady-state kinetics. To understand the reaction mechanism better, tests were also performed in duplicate following the ping–pong kinetics (Table 3) [13]. 'TableCurve 3D' software (Version 4.0, SYSTAT Software Inc.) was used to obtain the coefficients by fitting the data in the standard equations. The difference in the values of the kinetic constants obtained from both kinetics shows the effect of inter-dependence in a chain reaction, especially for the ABTS radical cation.

Table 2
Kinetic constants for CDH with lactose and ABTS radical cation as substrate following the steady-state kinetics

Substrate	Kinetic constants		
	K_m	K_{cat} (s^{-1})	Kinetic efficiency, K_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Lactose	1.43 mM (0.186)	2.877 (0.062)	2.012
ABTS radical cation	9.65 μM (1.34)	5.60 (0.141)	580.31

The values given in parenthesis denote the standard error.

Table 3
Kinetic constants for CDH with lactose and ABTS radical cation following the ping–pong kinetics

K_{cat}	0.583 (0.029)
K_m, lactose (mM)	0.35 (0.016)
K_m, ABTS (mM)	0.056 (0.008)

The values given in parenthesis denote the standard error.

3.4. Central composite design and electrolysis for optimisation

Twelve electrolysis tests were performed as shown in Table 1. Samples were collected at regular intervals for up to 24 h. Three control electrolysis tests were run (1st without CDH; 2nd without ABTS; 3rd without CDH and ABTS), under similar conditions to see if the oxidation of lactose could be achieved in absence of one or more intermediate reactions (Fig. 2). No conversion of lactose to lactobionic acid was observed at all.

The values of turnover frequencies are contained in Table 4, as an observed response against the corresponding electrolysis run. Additional data was provided to the software, where the ***tf*** value was assigned as zero for all the values of X and Y at zero. The corresponding predicted values were calculated by using the mathematical model derived from the coefficients of the model (Table 5).

The application of response surface methodology yielded the following regression equation, which is an empirical relationship between the turnover frequency (Z) and the molar concentrations of lactose (X) and the ABTS (Y) in the reaction mixture (Fig. 5).

$$Z = -41.7716 + 3.6029X + 1086.265Y - 0.0437X^2 - 4129.91Y^2 + 43.846XY$$

Table 4
Observed response and the predicted values by response surface fit for the corresponding experiment

Run no.	Turnover frequency (h^{-1})		Residual value
	Observed response	Predicted value	
1	345.60	347.42	−1.82
2	319.49	347.42	−27.92
3	315.89	347.42	−31.52
4	346.44	347.42	−0.98
5	296.86	174.47	122.38
6	350.20	397.81	−47.60
7	507.11	545.82	−38.71
8	169.45	190.95	−21.50
9	207.94	122.38	85.56
10	338.07	269.61	68.46
11	639.74	529.79	109.95
12	311.71	385.90	−74.19

Table 5
Model coefficients for the response surface fit estimated by multiple linear regression

Coefficient	Value	*t*-Value	*p*-Value
a	−41.7716	−1.77005	0.0872
b	3.6029	3.11935	0.0040
c	1086.265	1.92860	0.0636
d	−0.0437	−3.24894	0.0029
e	−4129.91	−1.17657	0.2489
f	43.846	7.76166	<0.0001[a]

[a] Significant at $p < 0.001$.

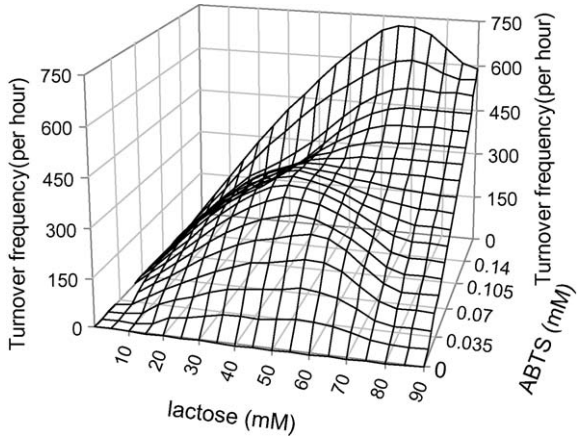

Fig. 5. Response surface plot showing the effect of millimolar concentrations of lactose and the ABTS radical cation on the turnover frequency for the biocatalyst (CDH) in the reaction mixture.

The significance of each coefficient was determined by Student's *t*-test and *p*-values (Table 5). The larger the magnitude of the *t*-value and the smaller the *p*-value, the more significant is the corresponding coefficient. This implied that the factor most significant (*t*-value = 7.76166, *p*-value < 0.0001) for turnover frequency was the mutual interaction term, or the relative concentrations of the substrate and the redox mediator with respect to each other.

The results of the second order response surface fitting in the form of analysis of variables are shown in Table 6. The Fischer *F*-test with a very low probability value ($p < 0.0001$) showed a very high significance for the regression model. The value of the determination coefficient ($R^2 = 0.9417$) verifies the suitable fit of the model, thus indicating a discrepancy of 5.83% for total variation, which is a normally accepted range of experimental error. The value of the adjusted determination coefficient (adjusted $R^2 = 0.929$) is also very high which indicates a high significance for the model.

Table 7
Observed turnover frequency for CDH with increasing concentrations of ABTS at 60 mM lactose in the reaction mixture

Run no.	Lactose (mM)	ABTS (mM)	***tf*** (h^{-1})
1–4	60	0.1	331.88
9	60	0.0293	207.94
11	60	0.1701	639.74
13	60	0.15	457.32
14	60	0.20	711.71
15	60	0.25	842.67
16	60	0.30	814.22
17	60	0.35	456.06

3.5. Further electrolysis tests

As observed from the regression analysis, the maximum turnover frequency for a fixed biocatalyst concentration in the reaction mixture could be obtained at the highest possible combination of the substrate and the redox mediator. This meant working around the solubility limits of lactose in the present buffer and perhaps at higher ABTS concentrations. Since it is regenerated continuously, there is no need to go up to the maximum.

From the data available, the solubility of lactose depends greatly on the water concentration in the solvent mixture and for 100% water at 25 °C the maximum solubility is reported at around 550 mM [14]. There are some variables such as temperature, turbulence, particle size distribution, impurities and dissolved lactose concentration, which may affect the rate of dissolution [15]. It was beyond the scope of this investigation to determine the solubility limits of lactose for the electrolysis buffer. Hence for interpretation purposes, the combined concentration was increased by raising the concentration of ABTS only (as very low concentrations are required) while keeping the lactose concentration constant at 60 mM. Further electrolysis tests were performed and the data obtained (Table 7) along with the previous data for 60 mM lactose were fitted in a new regression equation.

The fall in ***tf*** values with increasing ABTS concentrations could account for the fact that the ABTS radical cation when not reduced, is oxidised further to its di-cation, which is irreversible in the solution [4] and effectively fewer ABTS radical cations are available to oxidise the enzyme CDH which in turn oxidises

Table 6
Analysis of variance for the response surface fit to the experimental results of central composite design

Sources of variation	Sum of squares	Degrees of freedom	Mean square	*F*-statistics	*p* > *F*
Regression	1045553.1	5	209110.63	93.747	<0.0001
Residual	64686.79	29	2230.579		
Total	1110239.9	34			

$R^2 = 0.9417$, adjusted $R^2 = 0.929$.

Table 8
Observed turnover frequencies for CDH at higher concentrations of lactose and ABTS in the reaction mixture

Run no.	Lactose (mM)	ABTS (mM)	*tf* (h^{-1})
18	100	0.437	1443.51
19	150	0.656	1979.07
20	200	0.874	2075.00
21	250	1.093	2048.21
22	300	1.311	2062.62

lactose. The linear equation that fitted the data closely is

$$Z = 158.442 + 115489.53Y^{2.5} - 187805.52Y^{3}$$

where Z is the ***tf*** of the biocatalyst and Y is the millimolar concentration of the ABTS. The equation had a significantly high determination coefficient $R^2 = 0.9749$. The optimum value was 0.262 mM ABTS for 60 mM lactose which would give a turnover frequency of 845.61 h^{-1} for the biocatalyst. Electrolysis tests were performed in triplicate for these values and ***tf*** values of 841.42, 850.20 and 848.11 h^{-1} were obtained. The theoretical ***tf***, extrapolated from the kinetic tests for 60 mM lactose at the enzyme concentration used in the reaction mixture was 859.53 h^{-1}. All aspects considered, it could be stated that the optimised experimental results were quite close to the theoretically predicted values.

Subsequently, further electrolysis tests were performed with an increased concentration of lactose and ABTS (Table 8) in multiples of the optimised values obtained above. This was important for interpretation and comparative purposes.

The data obtained showed that the turnover frequency increased with the increase in concentration of substrate and mediator. However, the increase was not consistent and later a saturation in performance was observed. Also with 300 mM lactose the reaction mixture was not very clear, showing the solubility limitations in the present system. Nevertheless, the interpretation from response surface analysis was correct and the saturation in catalytic activity could be justifiably accepted for the amount of catalyst used.

3.6. Comparative analysis

The best productivity in this investigation was 1.77 g $(l\,h)^{-1}$ at 200 mM lactose and 0.874 mM ABTS. Noting that the amount of catalyst used was very low, it would be fair to consider specific productivity, which was 17.7 g $(h\,kU)^{-1}$ and is higher than the best result for the enzymatic regeneration method. Productivity can be increased by increasing the CDH concentration in the reaction mixture, thus reducing the total reaction time for the batch process. However, the results would be more promising if CDH concentration was higher in a repeated batch or a continuous process. With RSM it was possible to understand and improve the turnover frequency of the catalyst. However with limited resources, it was not possible to investigate the effect of experimental parameters such as: buffer concentration, temperature and working potential and these were kept constant for all the experiments. Nevertheless, these results ensure that with a commercial electrolysis set-up and better resources, this process could show far better results compared to other methods.

4. Conclusion and outlook

A process for the production of lactobionic acid from lactose via electrochemically regenerated flavoenzyme – cellobiose dehydrogenase – was successfully developed. The process, which showed better results than the enzymatic process for the same amount of CDH, was further investigated for higher turnover frequencies (***tf***s) for the biocatalyst. This work demonstrates the use of central composite design to determine optimum conditions. The results from this elementary study are very promising and could lead to a cost-effective and environmentally friendly process for the production of lactobionic acid. Further work is planned using a commercial electrolysis flow cell, where other factors such as buffer concentration, working potential and temperature could be investigated and optimised. In addition, continuous product removal by crystallisation is planned and batch mode electrolysis will be developed further for fed-batch or continuous processes.

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