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Short title:

Cost-estimation of enzyme use in SSF

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Highlights

Economic feasibility of immobilized enzymes in SSF has been investigated.

Magnetic CLEAs recycling from high solids loading SSF process is possible with good efficiency.

Applying immobilized enzymes is more viable for expensive enzymes.

SSF - Simultaneous saccharification and fermentation
m-CLEAs - magnetic Cross-linked Enzyme Aggregates
SSDF - Simultaneous saccharification, detoxification and fermentation

Abstract

Enzyme cost is considered to be one of the most significant factors defining the final product price in lignocellulose hydrolysis and fermentation. Enzyme immobilization and recycling can be a tool to decrease costs. However, high solid loading is a key factor towards high product titers, and recovery of immobilized enzymes from this thick liquid is often overlooked. This paper aims to evaluate the economic feasibility of immobilized enzymes in simultaneous saccharification and fermentation (SSF) of lignocellulose biomass in general, as well as the recuperation of magnetic immobilized enzymes (m-CLEAs) during high solid loading in simultaneous saccharification, detoxification and fermentation processes (SSDF) of lignocellulose biomass.

Enzyme prices were obtained from general cost estimations by Klein-Marcuschamer et al. [Klein-Marcuschamer et al. (2012) *Biotechnol. Bioeng*. 109, 1083–1087]. During enzyme cost analysis, the influence of inoculum recirculation as well as a shortened fermentation time was explored. Both resulted in 15 % decrease of final enzyme product price. Enzyme recuperation was investigated experimentally and 99.5 m/m% of m-CLEAs was recovered from liquid medium in one step, while 88 m/m% could still be recycled from a thick liquid with high solid concentrations (SSF fermentation broth). A mathematical model was constructed to calculate the cost of immobilized and free enzyme utilization and showed that, with current process efficiencies and commercial enzyme prices, the cost reduction obtained by enzyme immobilization can reach around 60 % compared to free enzyme utilization, while lower enzyme prices will result in a lower percentage of immobilization related savings, but overall enzyme costs will decrease significantly.

These results are applied in a case study, estimating the viability of shifting from sugar to lignocellulose substrate for a 100 t lactic acid fermentation batch. It was concluded that it will only be economically feasible if the enzymes are produced at the most optimistic variable cost and either the activity of the immobilized catalyst or the recovery efficiency is further increased.

1 Introduction

Lignocellulose based microbial production contains four subsequent steps: pretreatment, hydrolysis, fermentation and downstream processing. Hydrolysis is mainly done by different *cellulases* (Shahzadi et al., 2014), with enzymes alone taking a significant part of the overall production costs (Klein-Marcuschamer et al., 2012). Applying simultaneous saccharification and fermentation solves the product inhibition by glucose of the β-glucosidase enzymes and can increase the product yields. After thermal pretreatment, detoxification is often needed for better performance, as inhibitors (i.e., phenolics, furans and weak acids) can significantly hinder both hydrolysis and fermentation (Moreno et al., 2014). As the lignin-derived compounds have the highest toxicity, detoxification from phenolic inhibitors by *laccase* enzymes is considered a valuable solution as it is one of the few possibilities for process integration in simultaneous saccharification and fermentation, resulting in a simultaneous saccharification, fermentation and detoxification process (Jurado et al., 2009, Fonseca et al., 2013). *Laccase* is a copper-containing oxidoreductase that acts directly on phenols by oxidation. Radical formation and polymerization result in less toxic compounds (Soti et al. 2016). Addition of some types of *laccase* will even enhance the hydrolysis (Moreno et al., 2016, De La Torre et al., 2017) and thus lower the needed *cellulase* amount. To allow a cost effective downstream processing, industrially relevant titers (free

sugars and products alike) should be reached. This can only be achieved at high initial solid loading in enzymatic hydrolysis (Di Risio et al., 2011).

Enzyme prices are hard to estimate as it was detailed by Klein-Marcuschamer et al. (2012). There is so little information available, that often authors give an estimated price without indicating sources or models. These prices range from 3 $/kg up to 200-2000 $/kg (Brask et al., n.d.), with a theoretical minimum of 1.25 $/kg in case of soy protein (Klein-Marcuschamer et al., 2012). A model for price calculation of *cellulase* enzyme production from raw materials to final concentrated enzyme product was published (Klein-Marcuschamer et al., 2012). The model was based on available data and a simple process flow incorporating seeding the inoculum, fermentation, filtering, incinerating the cells and concentrating the enzymes to 150 g/l (also transport, taxes, wages etc. are included). The model estimates the production cost of *cellulase* from steam exploded poplar to be 10.14 $/kg in the base case, from which 49 % is facility dependent, 27 % the cost of raw material, and the rest includes utilities, wages, transportation, consumables and waste treatment. From raw material cost, glucose, NH_4OH and poplar are the dominant with a share of 33, 28 and 27 %, respectively. It is shown that the relative high cost of the enzyme itself is caused by both the expensive equipment and high operating costs (nearly 50-50 %). Excluding fixed costs, the price is still about 5 $/kg, which would be the case with a fully paid back production line. These estimations are useful as several enzymes could (and must) be used for a lignocellulose biomass based SSF process including different *cellulases* (*endo-*, *exocellulase*, *hemicellulase* etc.) and *laccases* (Soti et al., 2016).

A possible way to decrease enzyme cost is immobilization, which allows catalyst recycling by filtration or centrifugation (Sheldon, 2007), but also can increase the overall performance of the enzyme by improving the resistance to inhibitors and its general stability, widening the pH or temperature working range, or even achieving enzyme purification during the process (Garcia-Galan et al., 2011). However, also the immobilization step has its costs and while improved stability is experienced with enzymes that are used in organic solvents, such as immobilized lipase (Gao et al. 2013), for *laccase* and *cellulase*, enzyme deactivation is noticed due to the immobilization process (Liu et al. 2012, Bayramoğlu and Arica 2008, Liao et al. 2010, Alftrén and Hobley 2014). Moreover, during the application of immobilized enzymes, mass transport phenomena can cause a lower overall activity (Bhattacharya and Pletschke, 2014). Garcia-Galan et al. (2011) published a good overview about immobilization possibilities. Techniques can be divided in methods using support materials (porous or nanoparticles) or without a support, as in case of CLEAs (Cross-linked Enzyme Aggregates) or CLECs (Cross-Linked Enzyme Crystals), and these two types can even be combined. Therefore, applying the considerations published in the article to the specific case of simultaneous saccharification, detoxification and fermentation, the following facts can be deduced. (1) For *cellulase* catalyzed hydrolysis, the substrate is solid, and thus cannot penetrate into pores. Therefore, the enzyme has to be on or close to the surface. (2) Even after maximum theoretical conversion of the substrate, there will still be solid parts remaining in the broth (lignin particles, see Soti et al., 2016) which will hinder catalyst filtration. Therefore, separation has to be done based on a different principle. (3) During the process, the formed particles can block the porous structure of the carrier material. (4) The enzymes used are always mixtures and rarely purified to decrease the costs. As CLECs are formed after crystallization of pure enzymes, this technology is not applicable. (5) Production of CLEAs is based on aggregation of enzymes and can be used for our application. Based on these considerations, m-CLEAs (magnetic Cross-linked Enzyme Aggregates) are investigated further, as the carrier is non-porous, the enzymes are close to the surface and the separation can be done based on magnetic properties.

A significant amount of literature is available about magnetic CLEAs, or similar immobilization methods, from *laccases* or *cellulases,* however with varying success. *Laccase* was immobilized before on magnetically active mesoporous carbon with high enzyme loading (~50 m/m%) and relative high remaining activity (80-90 %), however even with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, a soluble substrate) it has lost 30 % and 50 % activity respectively (Liu et al., 2012). Horseradish peroxidase (similar to *laccase* enzyme) was immobilized on magnetic beads (~100 µm diameter) with a consecutive glutaraldehyde activation and immobilization. This led to low enzyme loading (~0.3 m/m%) but high retained activity (69 %) compared to free enzyme. However, immobilization improved working pH range, and thermal stability. Recirculation was not investigated (Bayramoğlu and Arica, 2008). *Cellulase* was immobilized on Fe_2O_3 nanoparticles (final catalyst size ~100 nm, 16 m/m% enzyme loading) with simultaneous cross-linking and immobilization. Remarkably, a high remaining activity (99 %) was measured with a soluble standard substrate, i.e. sodium carboxymethylcellulose, with a loss of only 8 % activity in 15 cycles and 33 % in 20. Also real substrate (corncob) was applied in a low concentration (<1 m/m%) and a high catalyst-substrate ratio (25 m/m%), where 62 % of hydrolysis yield was

achieved. Recirculation was not reported (Zhang et al., 2016). Liao et al. (2010) immobilized *cellulase* on polyvinyl alcohol /Fe_2O_3 (270 nm), the enzyme retained 91% filter paper activity. When they applied the catalyst on microcrystalline cellulose substrate at low concentration (2.5 m/v%) in a ball mill, it lost 60 % activity in four cycles. In these experiments the enzyme was not separated from the mixture, but the whole solid part was recycled (Liao et al. 2010). By Alftrén and Hobley (2014), *cellulase* was covalently immobilized on different paramagnetic particles (1 μm diameter, ~1 m/m% enzyme loading, ~50 % remaining activity). The catalyst was tested on pretreated wheat straw at low substrate loading (2 m/v%) and after magnetic recycling the activity decreased by 20 %.

Although these important improvements on the use of magnetic carriers in *cellulase* and *laccase* immobilization were reported in literature, they were rarely applied for lignocellulose biomass substrates, let alone for simultaneous saccharification and detoxification and high solids conditions.

Since our research addresses the simultaneous use of laccase and cellulase enzymes, co-immobilization can also be considered. In literature, some examples of co-immobilized enzyme systems exist. For example, Chmura et al. (2013) reported about combi-CLEAs. In this case several different enzymes are co-immobilized, although no cellulase or laccase is involved in the study. A more recent example on the co-immobilization of *lipase* and *galactosidase* was reported by Peirce et al. (2016), showing that even enzymes with different inactivation characteristics can be co-immobilized and the one faster deactivating selectively removed from the carrier. The stable enzyme was first immobilized on agarose support, thereafter the catalyst treated with ionic polymer and the second enzyme weakly immobilized via ion exchange. The latter can be removed selectively if it is deactivated. It should be noted that the applied *cellulase* complex is already a mixture containing a number of different enzymes. However, co-immobilization of the cellulase complex and laccase can be an interesting topic for further investigation.

In this paper, a laboratory scale m-CLEA enzyme recovery investigation is reported from a simulated fermentation broth with high solid content.

Techno-economic investigations on enzyme immobilization are rare, and none was found investigating the efficiency of recycling from an often difficult liquid such as high solid loading (10-20 m/m%) SSF fermentation broth. A published cost estimation (Borole and Davison, 2007) used a price of 900 $/kg for immobilized catalyst in a packed column process, which is a realistic pessimistic estimation (commercial price), while a lower estimate from Novozymes is 500 $/kg catalyst with 5 % enzyme loading (Brask et al. , n.d.). Both correspond to a specialty polymer as carrier material and relatively low enzyme loading.

The current paper researches the enzyme's possible contribution to the costs in the simultaneous saccharification, detoxification and fermentation of ligocellulose biomass. First, the production cost of *laccase* enzyme starting from the model of Klein-Marcuschamer is evaluated by using shorter fermentation times, as it would be expected in a bacterial process. Then, the possible decrease of enzyme price by inoculum recycling is investigated. Finally, a case study is presented estimating the cost of enzyme immobilization and recycling, and its effect on the enzyme cost during lactic acid production.

2 Methods

2.1 Enzyme price estimation was based on the model of Klein-Marcuschamer (http://econ.jbei.org) with some specific modifications made by using SuperPro Designer (Intelligen, Scotch Plains, NJ, USA). The downstream processing, taxes, wages, electricity, raw materials etc. were kept as was published. Modifications were made to investigate how the price would change if the inoculum was recycled or the fermentation time changed. The obtained estimation was used later to define the possible enzyme price range.

2.2 Experiments on immobilized enzyme recovery of m-CLEAs *laccase* from simultaneous detoxification and saccharification reaction mixture were performed. The immobilized enzyme was a kind gift from CLEA Technologies (Delft, NL), with a thermostable bacterial *laccase* from MetGen (Kaarina, Finland), an organic loading of 38 m/m% and based on glutaraldehyde cross linking from the crude *laccase* product. Immobilization was performed as described in Lopez et al. (2002) and Sheldon et al. (2016). No more information was communicated. The experiments were solely focused on the recuperation possibilities of the m-CLEAs from a thick liquid. Therefore, recuperation efficiency was based on m/m%. Recovery experiments were performed using an LH Fermenter 500 (LH Fermentation ltd., Reading, UK) and a neodymium magnet (4x2x0.4 cm). The

reaction mixture consisted of 12 m/m% steam exploded poplar (Soti et al., 2013), 0.13 m/v% organic loading equivalent m-CLEAs (19.1 g wet catalyst) and 2 m/v% *cellulase* liquid product (0.24 m/m% dry load) (Optimase CX15L, DuPont, Wilmington, DE, USA). The reactor setup (Figure 1) was modified by attaching metal rods on the rotor for high viscosity mixing. m-CLEAs were separated from the bioreactor after 24 h of hydrolysis when the broth was liquefied, i.e., converted from wet solids into a thick liquid. Hereto, the magnet was attached to the rotor, stirring speed linearly decreasing from 500 to 0 rpm in 30 min. In this way, it was expected that higher agitation rates caused better mixing and thus improved the contact of the catalyst to the magnet, but also intensify the disturbance of the attached catalyst layer as the catalyst remaining on the magnet is a mixture of iron, enzyme and lignocellulose particles, thus the magnetic field is weaker layer by layer. Lower agitation rates would result in less layer disturbance but also less contact and mixing. The optimum agitation rate was not determined, but it was expected that by linearly decreasing the rpm over a long time the agitation rate will go through the optimum (collect the most catalyst possible), and on a lower value it will maintain the collected material as the breakaway effect is decreasing. Afterwards the material containing the catalyst was released from the magnet, washed in 200 ml demineralized water and subsequently separated from the washing liquid by the magnet again. The mass of the remaining material was measured. This washing cycle was repeated 16 times, i.e., until the washing liquid was transparent (all lignocellulose material was washed out). Two parallel experiments were performed.

2.3 A case study was performed for estimating the enzyme cost for 100 t lactic acid fermentation, with 0.1 m/v% (1 g/l) purified high activity *laccase* equivalent industrial enzyme loading (1 m/v%, 10 g/l). To quantify the trade-off between the extra cost of free enzyme and the cost of immobilization and reuse, equations were constructed. Further calculations were based on this model. Recycling efficiency values (obtained from experiments detailed in **Section 2.2**) and enzyme deactivation (not published results), were used to estimate the immobilized enzyme utilization costs. The impact of recycling efficiency was evaluated at three levels: 0.8, 0.9 and 0.99, keeping the deactivation ratio during immobilization constant at 0.5. The impact of enzyme deactivation was also investigated at three levels, i.e., 0.5, 0.3 and 0.1 while recycling efficiency was kept at 0.8. Best case scenario, using 0.1 enzyme deactivation and 0.99 recycling efficiency, was compared to the worst scenario with 0.5 and 0.8 respectively.

All data processing was performed off-line using a commercial software package (MATLAB 2017.1, The MathWorks Inc., Natick, MA, 2017). Prices applied during cost calculations were taken from Alibaba ("Manufacturers, Suppliers, Exporters & Importers from the world's largest online B2B marketplace-Alibaba.com," n.d.): rotating drum magnet, slurry pump, iron powder, glutaraldehyde, lactic acid. An industrially relevant lactic acid concentration was taken from published data (Zhao et al., 2013).

3 Results and discussion

3.1 Enzyme price. The previously mentioned model (Klein-Marcuschamer et al., 2012) was used as an estimation of the general costs for extracellular enzyme production. Parameter sensitivity was investigated to see how the price would change in case of different enzyme production. It has to be noted that the model is based on wood degrading microorganisms, as poplar is one of the carbon sources.

Assuming 24 h fermentation, instead of 192 h, or 96 h in case of seed tanks, would decrease the production cost of *cellulase* from 10.14 $/kg to 8.65 $/kg, with 32 % raw material related costs and 48 % facility dependent (1.5 $/kg decrease). Switching to bacterial fermentation usually shortens fermentation time, however 24 h is optimistic. It can be seen that 8 folds of decrease in fermentation time only leads to 15 % drop in production cost.

It is also not easy to decrease raw material costs for enzyme production. One possibility is to increase the yield of enzyme production, since this way less carbon source would be used per unit of enzyme produced. This would also decrease fermenter and downstream processing costs at the same time. However increasing enzyme yield further will be difficult because calculations are already done based on an optimized *cellulase* production process. The second possibility is to recycle the inoculum, which would mean technically substituting the glucose carbon source that is applied for culturing the inoculum with cheaper poplar. In the meantime it would make seed tanks unnecessary, which represent around 9 % of CAPEX. This way the production price was decreased from 10.14 to 8.63 $/kg, from which 51 % is facility dependent and 21 % raw material dependent. Altogether this means 14.9 % decrease in final price. From the raw materials part, poplar and ammonium hydroxide together take around 40 % of the costs. In this remaining production price, the raw

materials account for 1.81 $/kg and 4.3 $/kg is assigned to all variable costs, which can be considered as very low. Issues, such as the seeding of the fermentation tank once at the start of the first fermentation or genetic stability of the microorganisms were not taken into account.

It can be assumed that enzyme prices will not be lower than the cheapest protein on the market, i.e. soybean, which is around 1.25 $/kg (Klein-Marcuschamer et al., 2012), and not higher than the current price, which is 200-2000 $/kg for bulk enzymes offered by Novozymes (Brask et al., n.d.). For *laccase* enzyme, no price estimation models are available. The general ideas about the *cellulase* prices apply for *laccase* too, so the investigation of process costs in **Section 3.5** has been conducted in the mentioned regime (1.25-200 $/kg) in order to give an overview on the whole possible range.

3.2 Constructed model for cost estimation of immobilized enzyme systems
The cost of enzyme is simply the multiplication of the mass of the fermentation batch [t], the concentration of enzyme in the batch [kg/t] and its price [$/kg].

$$Cost_{free} = Volume \times Concentration_{enzyme} \times Price_{enzyme} \qquad (1)$$

The calculation of enzyme cost including immobilization can be written in a similar way as in Equation (1) with the difference that the catalyst price is the addition of enzyme and immobilization price (calculated in $/kg_{protein}$), and the enzyme amount has to be recalculated with the deactivation caused by immobilization. Recycling reduces the cost as only the catalyst that could not be recovered has to be replenished to allow the same enzyme activity for each batch. The added amount in each round of reuse is the catalyst that could not be recycled, i.e. *(1-Recirc)*. This amount has to be corrected with one factor, reflecting the enzyme loss and deactivation during the immobilization process, i.e. $Deact_{immob}$, which was taken to be 50 % in the worst case scenario of the case study. However in some reports this deactivation was measured to be only 10-20 % (Bhattacharya and Pletschke, 2014). It is very likely that the enzyme stability and the pH working range are improved after immobilization. This will also be incorporated in the variable $Deact_{immob}$, expectedly decreasing it because inhibitors present in the hydrolysate can also inhibit enzymes (e.g. inhibition of *cellulases* by phenolics), thus the immobilization can grant the enzyme better inhibitor resistance. The pH applied during SSF can differ from the optimal pH of the enzyme; again immobilization can yield in less activity loss caused by the pH shift.

$$Cost_{immob} = Volume \times Concentration_{enzyme} \times (Price_{enzyme} + Price_{immob}) \times \frac{1-Recirc}{1-Deact_{immob}} \qquad (2)$$

The savings achieved at each cycle by immobilization is the subtraction of the two (Equation (1)- Equation (2)), normalized by the cost of free enzyme treatment (Equation (1)).

$$Saving = 1 - (1 + \frac{Price_{immob}}{Price_{enzyme}}) \times \frac{1-Recirc}{1-Deact_{immob}} \qquad (3)$$

Equation (3) will be applied to quantify the savings factor achieved by immobilization in Sections 3.5.

By investigating Equation (3), some general remarks can already be given, i.e., the saving is higher if the ratio of immobilization price to enzyme price is lower, if the recycling efficiency ($Recirc$) is higher or if the deactivation by immobilization ($Deact$) is lower. Furthermore if the recycling efficiency is not higher than the loss by deactivation, the process cannot be cost effective under any circumstances.

An important remark on the application of these equations is that instead of deactivation of the enzyme during immobilization, the enzyme can obtain an increased activity, due to the favorable environmental conditions. In that case, the factor $Deact_{immob}$ will be a negative number.

3.3 Immobilization cost. The price for immobilization of m-CLEAs was estimated to be 5 $/kg protein, as the iron powder would cost 1-1.2 $/kg and the glutaraldehyde solution 2 $/kg. As the catalyst contains approximately 40 m/m% organic loading the mass corresponding to 1 kg of protein is 2.5 kg catalyst, containing 1.5 kg iron, if the mass of glutaraldehyde is neglected. The iron itself corresponds to 1.5-1.8 $/kg. A total cost of 5 $/kg was assumed, which most probably covers the variable costs.
Rotating drum magnets are available between 5000 and 50000 $, with 100 t/h capacity for the latter. They are equipped with a 3-5 kW motor (0.20 $/kWh, around 0.6-1 $/h electricity cost). For a 100t/h capacity equipment

and even if the liquid is recycled several times, the electricity cost is still under 100 $. The slurry pump price is around 5000 $ and energy consumption is also negligible. In Section 3.5, it will be shown that both CAPEX and consumptions are negligible compared to enzyme prices.

3.4 Recycling efficiency of *laccase* m-CLEAs. It is common that yields of only 20-30 g/l reducing sugars per 100 g wood are obtained after hydrolysis of pretreated wood (Wang et al., 2012). Therefore, the solid content after hydrolysis is still significant. During the simultaneous saccharification and detoxification experiments in our laboratory, it was observed that the initial wet solid fermentation mixture was changed into a smooth thick liquid after 12-24 h. m-CLEAs recycling was performed from demineralized water as a standard and from SSF hydrolysis medium as a possible industrial substrate. Enzyme recovery has been expressed in m/m% because of the adsorption of phenolics on the CLEAs, which hampers the measurement of the decrease in phenolics due to laccase activity separately from the decrease due to adsorption phenomena. Results of the recovery showed that the catalyst could be easily recycled from demineralized water with 99.5 m/m% efficiency, but 88 m/m% of the immobilized catalyst could be still recovered even from the thick liquid containing remaining lignocellulose solid particles (16 washing cycles were required to separate the catalyst from the solid part). Figure 2 shows the decrease in mass of the separated m-CLEAs catalyst, including some lignocellulose material, during the washing cycles. It has to be noted that this washing is only needed for analytical purposes. In an industrial application the separated unwashed catalyst will directly be reused in the next batch. The mass of recovered catalyst before washing was almost double that of the initial mass because of the presence of lignocellulose slurry that was removed from the reactor at the same time (see Figure 2). It can be seen that the final catalyst recovery from the broth was 88 m/m% after only one separation step.

3.5 Case study. Applying the constructed model and data obtained in previous sections, the economic feasibility of immobilized enzyme application is investigated. Assuming a 100 t batch fermentation of lactic acid (2 $/kg selling price) with 10 m/m% final product content, which is an industrially relevant titer, the whole value of the product in the reactor is 20 000 $. Calculating with 100 % yield (theoretical maximum), the price of used glucose is 4750 $/batch ("Sugar, European import price - Monthly Price - Commodity Prices - Price Charts, Data, and News - IndexMundi," n.d.). Poplar wood has in average 70 % carbohydrates, assuming full conversion (avoiding that pentoses could be only fermented with much lower yield), and a market price of 60 $/ton (Perrin et al., 2008), the gain would be 3900 $/batch compared to glucose based fermentation. This value must cover all the additional process steps (pretreatment) and materials (enzymes) caused by switching to lignocellulose from simple sugar.

Figure 3 shows the estimated free and immobilized enzyme prices per batch in function of selling prices of free enzyme [$/kg] and also visualises the saving factor due to the use of immobilized enzymes, as presented in Equation (3). Data from the literature survey on the effect of immobilization on laccase and cellulase in **Section 1** and from our own results were applied to define a worst case and a best case scenario. For the worst case scenario deactivation of 0.5 and recirculation of 0.8 were taken, while for the best case scenario the best reported deactivation (0.1) (Bhattacharya and Pletschke, 2014) and the recycling efficiency close to that obtained from demineralized water (0.99), which is the theoretical maximum, were used. Recirculation will become theoretically viable if savings factor is higher than zero (see Figure 3b), i.e. from an enzyme price of over 3.2 $/kg in worst case scenario. In practice this enzyme price value will be higher as the cost of recycling and deactivation during recycling were not considered. At 10 $/kg, which is an optimistic realistic enzyme price, the recycling would save 40 % or 4000 $/batch (see Figure 3a). This would pay back the whole recycling investment in a few dozens of cycles, which corresponds to a few months in case of fermentations that last for a few days. At higher enzyme prices the difference is even more remarkable, saturating at 59 % saving, which comes out from the mathematical relationship (Equation 3). In the best case scenario immobilization is viable at any realistic enzyme price and the saving saturating around 0.99.

Figure 4 presents the savings with respect to enzyme price at three different recycling efficiencies and a constant deactivation value of 0.5. The savings factor attains a zero value if the immobilized enzyme cost is the same as free enzyme utilization cost. This break-even value is obtained at enzyme prices of 3.2, 1.35 and 0.1 $/kg$_{protein}$ for 0.8, 0.9 and 0.99 recirculation respectively. Savings factors are saturating at 0.59, 0.80 and 0.98 respectively. The last recovery value is rather optimistic for the high-solids medium applied during simultaneous saccharification and fermentation processes, but 0.9 seems realistic based on experimental results where 0.88 was obtained. A 10 % increase in recovery efficiency (from 0.8 to 0.9) resulted in a

corresponding 36 % savings factor improvement. CLEAs utilization is a new topic in research, it is possible that even higher recirculation rates can be achieved by a well designed process.

Figure 5 shows the savings in function of enzyme price at three deactivation ratio levels and a 0.8 recycling efficiency. Investigating the influence of the deactivation values of 0.5, 0.3 and 0.1 on the savings factor, it can be concluded that the break-even cost is at enzyme prices of 3.2, 2.0 and 1.4 $/kg$_{protein}$ respectively and savings factors are saturating at 0.59, 0.70 and 0.77 respectively. In comparison to the experimental enzyme deactivation value of 0.5, the value of 0.1 will be hard to achieve. It corresponds to an 80 % decrease while the saving factor improved only by 30 %.

On the other hand it should be noted that the 3900 $ gain in substrate price if poplar wood is used instead of glucose (as mentioned before) should include all necessary extra steps, such as hydrolysis and detoxification. This would only fit in the case of the 3-3.5 $/kg free enzyme cost at break-even cost, and cover most of it (3000-3200 $/batch). At a price of 10 $/kg, as estimated by Klein-Marcuschamer et al. (2012), the cost of free enzyme alone corresponds to 10000 $ (Figure 3a), which is half the value of the overall product in the batch, and much more than what is saved by switching to poplar instead of glucose.

In the following reasoning the calculated enzyme costs of the different scenarios will be compared to each other at the two mentioned enzyme prices, i.e., 3.5 and 10 $/kg$_{protein}$. It has to be noted that for realistic application, the total enzyme cost should be under 3900 $, preferably making up maximum one third of the cost, while the free enzyme cost would already be 3200 and 10000 $/batch respectively. In the range of 3-3.5 $/kg enzyme price, the worst case scenario will only reach zero savings, indicating that immobilization will not be beneficial. At 10 $/kg price however, the enzyme cost is 6000 $, which is a 40 % reduction compared to free enzyme utilization. Best case scenario would mean an enzyme cost of 100 and 170 $/batch at 3.5 and 10 $/kg enzyme price respectively, which is very attractive, but unrealistic. **Improving the recirculation efficiency** to 0.99 or 0.9 from 0.8 (Figure 4) would reduce enzyme costs from 3400 to 170 or 1700 $/batch, respectively, in case of 3.5 $/kg$_{protein}$ enzyme price and from 6000 to 300 or 3000 $/batch, respectively, in case of 10 $/kg$_{protein}$ enzyme price. **Decreasing deactivation ratio** to 0.1 or 0.3 from 0.5 (Figure 5) would reduce enzyme costs from 3400 to 1890 or 2400 $/batch, respectively, in case of 3.5 $/kg$_{protein}$ enzyme price and from 6000 to 3300 or 4300 $/batch, respectively, in case of 10 $/kg$_{protein}$ enzyme price. These data show that increasing recirculation efficiency to at least 0.9 will be crucial for viable immobilized enzyme utilization in the investigated process. Any decrease in deactivation ratio would be beneficial but alone it is not enough to solve the problem, especially at realistic 10 $/ kg$_{protein}$ enzyme prices. Only in case of 3.5 $/kg enzyme for all investigated efficiencies, and in case of a deactivation ratio of 0.1 and a recovery efficiency of 0.9 or 0.99 at 10 $/kg enzyme price, the total enzyme cost will be under 3900$. A total enzyme cost under one third of the substrate profit, i.e. 1300 $, is rather unrealistic and will only be obtained in cases with 0.99 recovery efficiency.

As discussed in Section 3.1, reaching enzyme prices under/around 5 $/kg is possible if only the operating costs are considered. Producing at this price would hinder companies from investing, except maybe if it is a strategic field or if governmental funds cover the costs. As shown the variable costs could be decreased by shortening fermentation time or recycling the inoculum (among others, such as increasing enzyme activity and carbon yield). However, the case study indicated a needed enzyme price of 3-3.5 $/kg or unrealistically high catalyst recovery values to fit in the cost reduction gained by switching to poplar instead of glucose in a lactic acid fermentation. Additionally unrealistic lactic acid conversion rates were used: 100 % for both glucose and xylose. This estimation only includes *laccase* price and not *cellulase*. This altogether indicates that the market viability of lignocellulose based lactic acid is questionable even on theoretical level.

4 Conclusion

The driving force of enzyme recycling is the difference between the enzyme price and the immobilization price. The current enzyme prices are far from the calculated minimum. The latter is based on published cost estimation models for enzyme production and analysis of possible model modifications. If the price of the free enzyme is increased, the savings by enzyme immobilization will saturate around 60 % with current technology, while lower enzyme prices decrease the theoretically achieved savings. The observed possibility to recover m-CLEAs from thick liquid is remarkable but high enzyme prices hinder application in second generation processes with lignocellulose substrate. A case study investigating the shift from sugar to poplar wood (lignocellulose) raw materials for a 100 t lactic acid fermentation batch shows that it is economical feasible if the enzymes are

produced at the most optimistic variable cost. This can indicate that reaching a competitive commercial price for poplar derived lactic acid is impossible under the current market conditions.

It can be concluded that more research is necessary before commercial use of immobilized enzymes in simultaneous saccharification and fermentation of lignocellulose biomass will be economically viable. A lot of directions in research are possible to achieve this goal, e.g., the use of more efficient immobilization technologies such as magnetic nanoparticles can increase the enzyme activity and stability, which will both decrease the cost as less enzyme will be necessary. Enzyme activity and stability can be further increased by using other enzyme sources delivering more stable and efficient enzymes, or microbial strains producing higher enzyme titers, thus lowering downstream processing costs. Also future research on the co-immobilization of laccase and cellulase enzymes is an unexplored but interesting topic.

Conflicts of interest: none.

Contributors

Valentin Sóti – Experiments, Matlab modeling, cost estimation, writing

Silvia Lenaerts – PhD supervisor- advice

Iris Cornet – PhD supervisor, advice & co-authoring

All authors have approved the manuscript.

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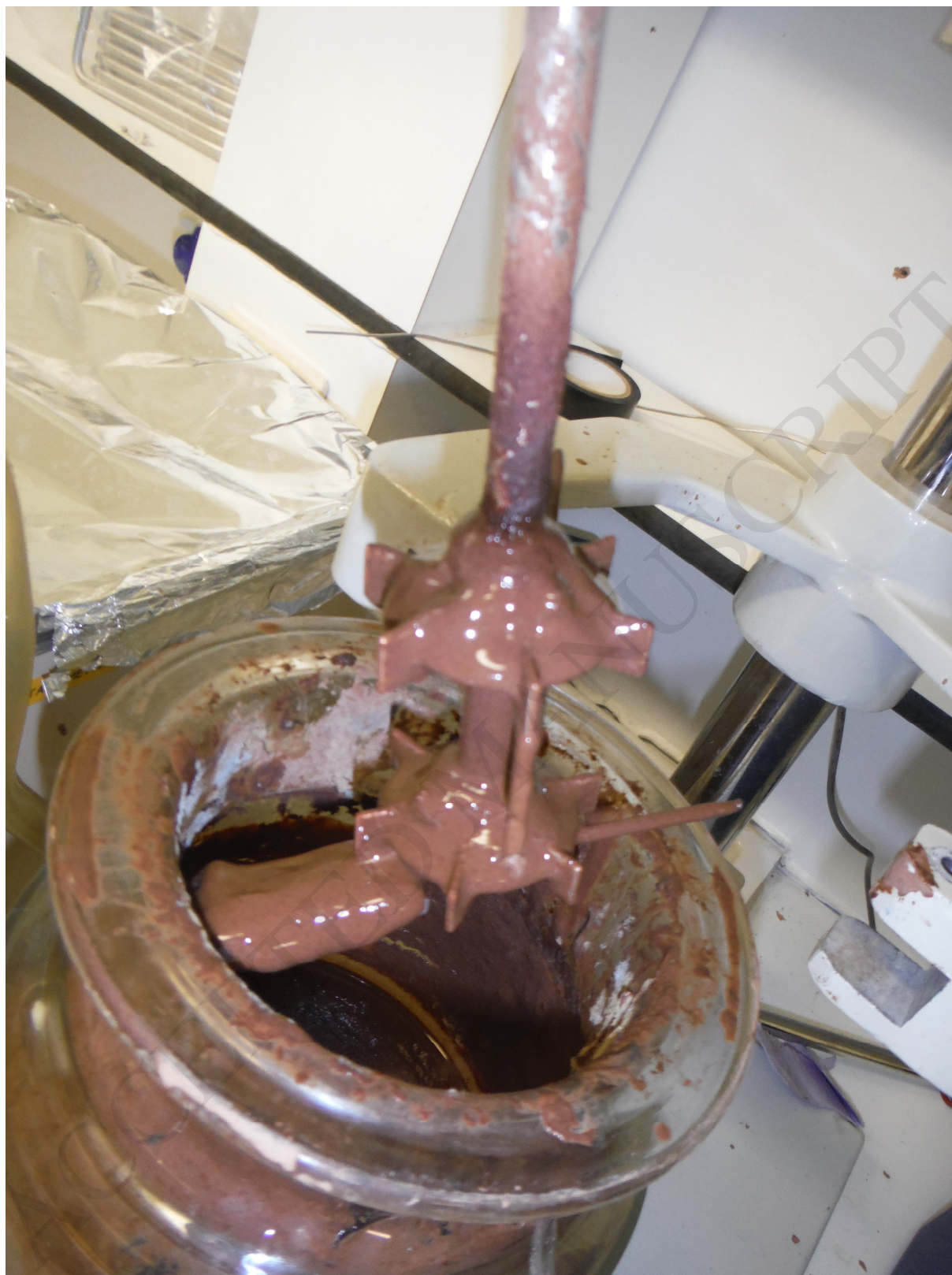
Figure 1: The set-up used for recovery measurements. The magnet is attached to the rotor.

CLEA separation from SSF

| Tara [g]	17.7511		Brutto [g]	Netto [g]	Fraction [-]	Theoretical
	Start	0	23.6459	5.8948	1	1
	Washing	1	28.8957	11.1446	1.890581529	1
		2	25.7476	7.9965	1.356534573	1
		3	24.689	6.9379	1.176952568	1
		4	24.2516	6.5005	1.102751578	1
		5	24.1742	6.4231	1.089621361	1
		6	24.1741	6.423	1.089604397	1
		7	23.813	6.0619	1.028347018	1
		8	23.7681	6.017	1.020730135	1
		9	23.559	5.8079	0.985258194	1
		10	23.469	5.7179	0.9699905	1
		11	23.4305	5.6794	0.96345932	1
		12	23.3513	5.6002	0.95002375	1
		13	23.2486	5.4975	0.932601615	1
		14	23.125	5.3739	0.911633982	1
		15	23.018	5.2669	0.893482391	1
		16	22.9599	5.2088	0.883626247	1
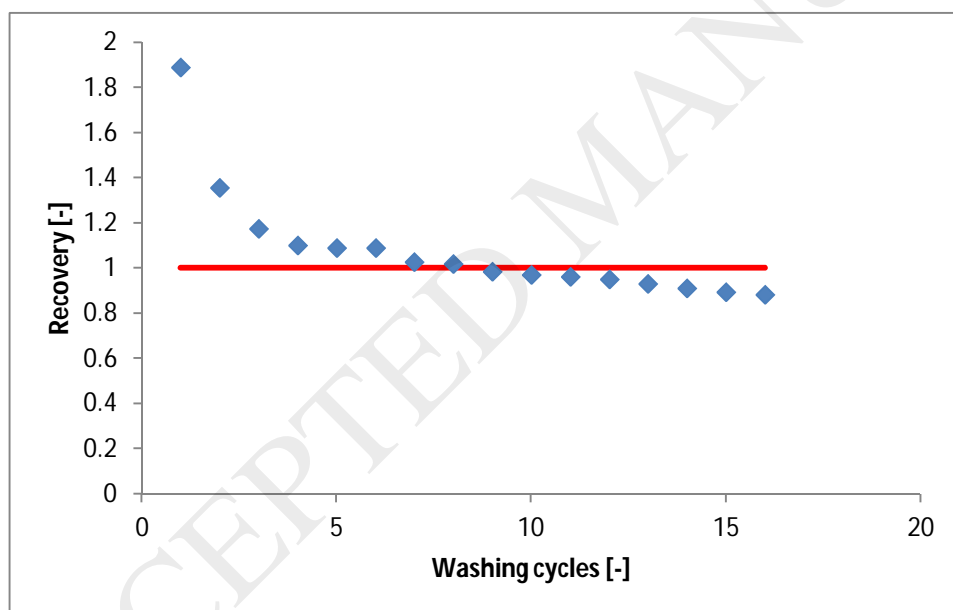
Figure 2: Mass recovered by magnet in function of washing cycles (♦) and line indicating the mass of initial m-CLEA (-).
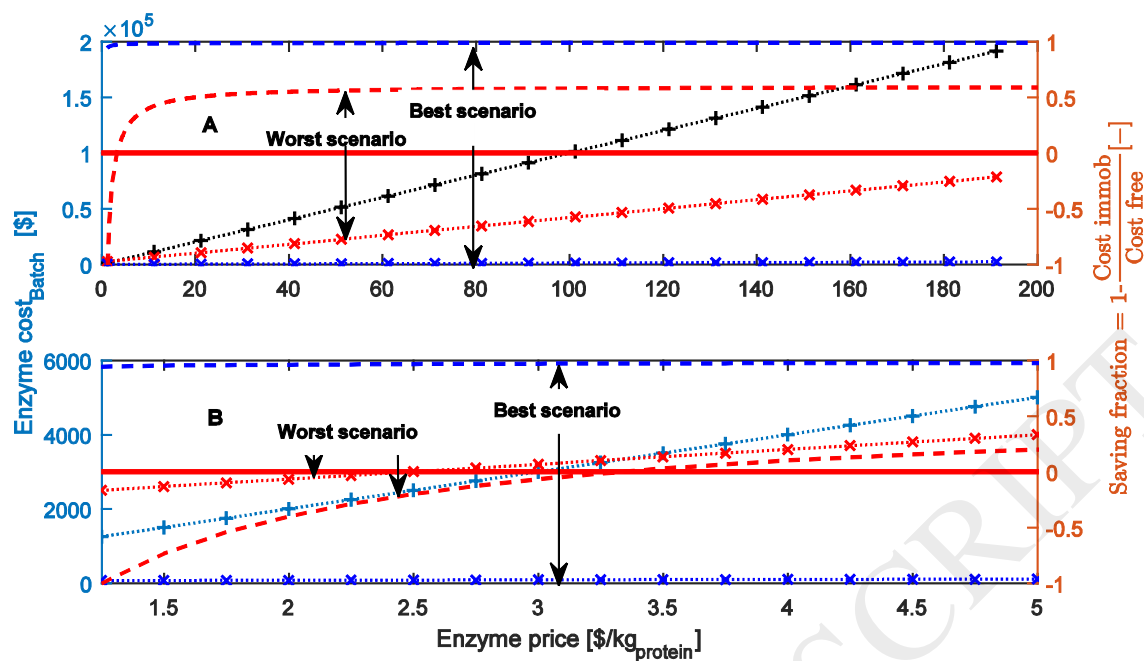
Figure 3: (A) Enzyme costs and savings in function of enzyme price. The cost of free enzyme per batch is marked with plus and dotted line (.+), the cost of immobilized enzyme with cross and dotted line (.x), the saving factor with dashed line (--), the 0 saving factor with full line (-).Worst case scenario corresponds to 0.8 recirculation efficiency and 0.5 deactivation ratio, best case scenario to 0.99 and 0.1 correspondingly. (B) Detail from upper graph.
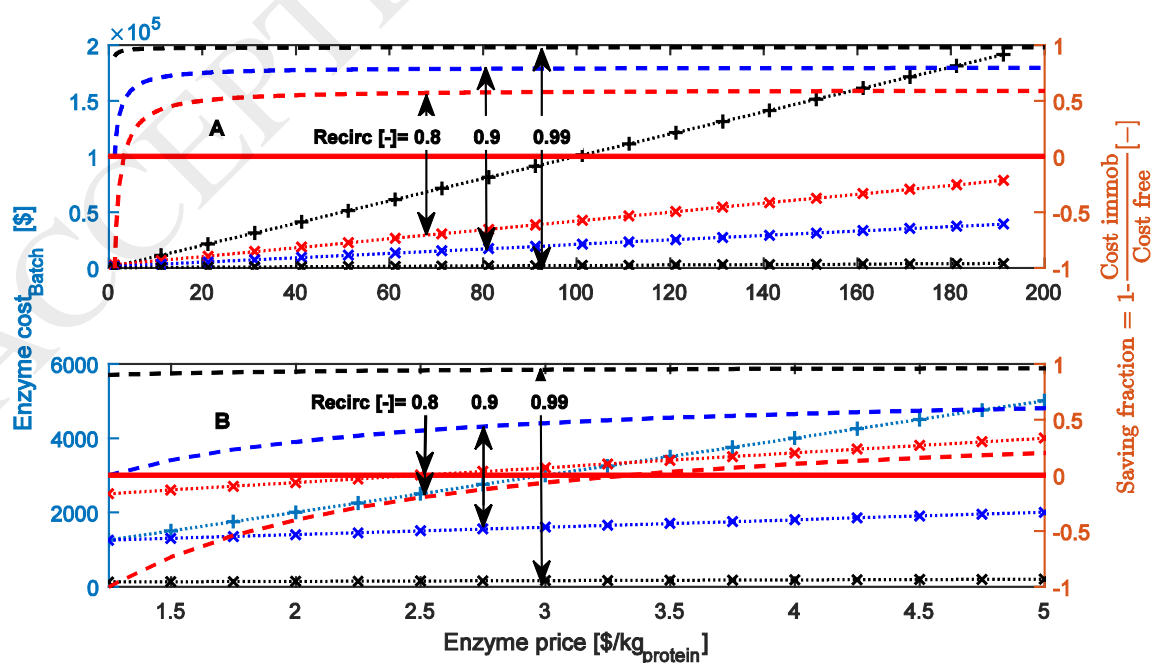
Figure 4: (A) Enzyme costs and savings in function of enzyme price at different recirculation efficiencies (0.8, 0.9 and 0.99) and fixed deactivation ratio (0.5). The cost of free enzyme per batch is marked with plus and dotted line (.+), the cost of immobilized enzyme with cross and dotted line (.x), the saving factor with dashed line (--), the 0 saving factor with full line (-). (B) Detail from upper graph.
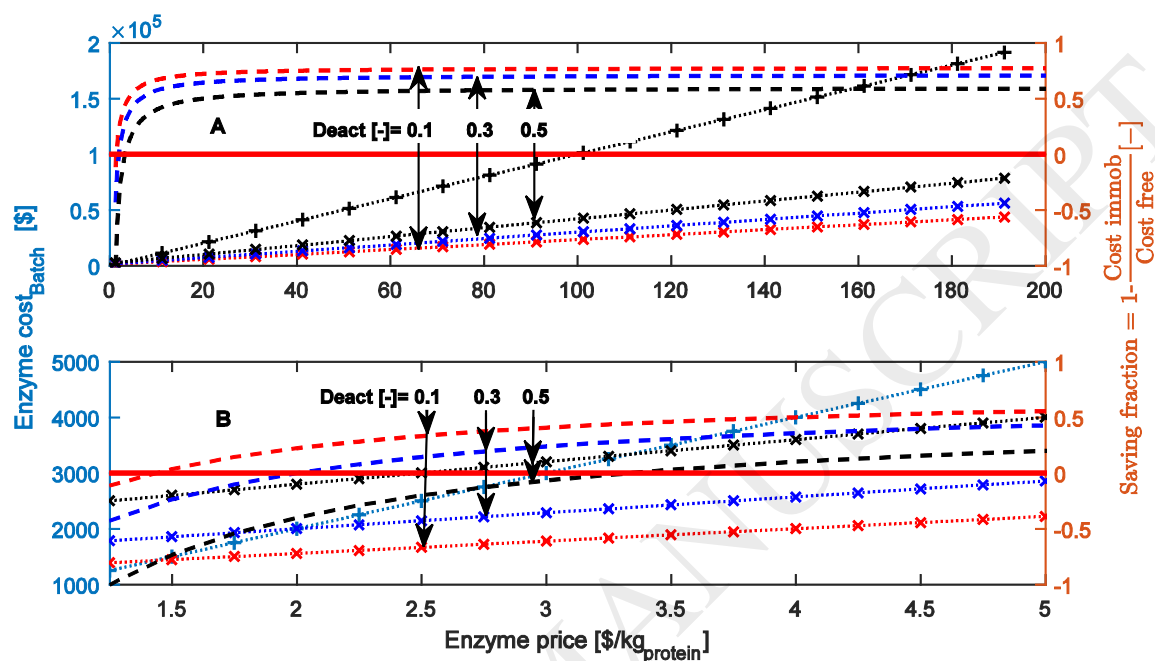

Figure 5: (A) Enzyme costs and savings in function of enzyme price at different deactivation ratios (0.1, 0.3 and 0.5) and fixed recirculation efficiency (0.8). The cost of free enzyme per batch is marked with plus and dotted line (.+), the cost of immobilized enzyme with cross and dotted line (.x), the saving factor with dashed line (--), the 0 saving factor with full line (-). (B) Detail from upper graph.