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Process simulation-integrated optimization of lignocellulolytic enzyme production

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HIGHLIGHTS

- *P. sanguineus* growth conditions are optimized for Lac, Bet, and Cmc activities.
- A novel workflow integrating experiments and process simulations is offered.
- Kriging is used to model time-profile data from activity measurements.
- The workflow is demonstrated on a bioethanol production process case study.
- Significance of Bet activity favors yeast extract dominated optimal growth medium.

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ABSTRACT

An optimization workflow is introduced which integrates multi-objective optimization of lignocellulolytic enzyme cocktail ingredients with a bioethanol production process where the enzymes are utilized. The workflow integrates data collection via exploratory experiments, modeling via Kriging, Pareto-based multi-objective optimization, and process simulation. The critical links in the integration are calculation of enzyme cocktail performance and cost. This allows the identification of the best Pareto-optimal result depending on process simulation results. The workflow is demonstrated on a case study involving the production of lignocellulolytic enzymes laccase, β-glucosidase, and carboxymethyl cellulase by a white rot fungus, *Pycnoporus sanguineus* DSMZ 3024. Concentrations of various carbon and nitrogen sources and culture duration are optimized. Two cases are analyzed: i) where all culture conditions and three enzyme activities are assumed to affect enzyme cost and performance equally; ii) where culture duration and β-glucosidase activity are assumed to respectively affect enzyme cost and performance more significantly compared to the other factors. The integrated optimization workflow identified a shift from a malt extract dominant growth medium in the first case to a yeast extract dominant medium in the second. This shift could not have been identified without the proposed workflow.

1. Introduction

Production of bioethanol from lignocellulosic feedstock is a promising and potentially sustainable alternative to petroleum-based fuel [1]. Enzymatic bioprocessing of lignocellulosic raw materials is a preferable route compared to alternatives like acid treatment, due to milder operating conditions and simpler downstream processing requirements [2]. However, the high cost of lignocellulolytic enzymes is a major challenge towards the commercialization of such bioprocesses [3]. Techno-economic analyses of bioethanol production processes from various lignocellulosic raw materials illustrate this situation. Enzyme cost contribution to bioethanol selling price has been shown to be as high as 48% [4] when corn stover is used as feedstock and around 13% on average [1]. Others have shown enzyme cost to have a considerable effect on operating costs varying around 16 [5] to 19% [6], when the feedstock is wheat straw and miscanthus, respectively.

As a way to reduce enzyme cost, enzyme improvement for better biomass conversion has been and still is a critical research priority [7,8]. Screening and selection of efficient organisms [9] and subsequent optimization of culture conditions for enzyme production [10] are routinely employed approaches towards this goal. Generally, the objective of optimization is achieving maximal lignocellulolytic enzyme activities [7] and preparing an optimal enzyme cocktail which lead to maximum reducing sugar content to be released from the lignocellulosic feedstock for subsequent bioethanol fermentation [11–14]. It is common practice in this area of research to limit the scope of

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publications to report optimal conditions and achieved activities. For example, Prasher and Chauhan (2015) studied the effect of various carbon and nitrogen sources on lignocellulolytic enzyme production by *Dictyoarthrinium Synnematicum* Somrith where optimal conditions were found for laccase (Lac), manganese peroxidase, and lignin peroxidase [15]. In another study, carboxymethyl cellulase (Cmc) activity was optimized via the consideration of growth medium ingredients such as peptone and yeast extract [16]. More recently, effect of medium ingredients and growth conditions were investigated with a statistical analysis of achieved FPase, Cmc, and β-glucosidase (Bet) activities [17].

The effect of optimal enzyme production conditions and achievable activities on overall bioethanol production techno-economics is not trivial. A quantitative and computational scheme to relate enzyme production optimization to bioethanol production process design is imperative. It is crucial to realize that enzyme improvement is only one aspect of a multi-faceted and interactive process design and development effort for lignocellulose-derived bioethanol production. Other aspects include feedstock pretreatment, fermentation, and downstream processing. So, enzyme improvement must be considered within a larger context including the conceptual design, modeling and simulation of the complete bioethanol production process utilizing these enzymes [4,18].

Often, bioethanol process design studies incorporate enzyme cost and performance as static assumptions [6], specified targets [19] or uncertain variables [20]. There isn't a dynamic link in any of these examples or similar studies that could computationally propagate the effects of enzyme production optimization on the techno-economics of bioethanol production. The authors of this paper observe a lack of dynamic integration between enzyme improvement and process design workflows. Therefore, a demonstration of concept level workflow is presented in this paper that entertains the idea of process simulation-integrated optimization of lignocellulolytic enzyme production.

In the conventional workflow, once a potential lignocellulolytic enzyme producing organism is identified and characterized, optimization of culture conditions of the selected organism is performed first and independently [21]. Later and separately, results from the enzyme optimization step (preferably quantified in terms of maximum enzyme activities achieved and estimated cost of enzyme production) are used within the conceptual design, modeling, and simulation of a bioethanol production process [5]. This approach can result in a sub-optimal situation since there is no dynamic interaction between experimental results and bioprocess simulations. Without such interactions, marginal improvements in enzyme activities achieved via standalone optimization in the first step may fail to allow feasible bioethanol production in the second step. For instance, a certain set of optimal culture conditions for enzyme production may marginally outperform another set of conditions. However, a possible corresponding elevation in enzyme production costs may be too high to justify the preference of the marginally better option. To quantify the trade-off, cost and performance of the enzymes should be contextually related. A more holistic approach should integrate the effects of changes in enzyme production conditions with the techno-economic performance of the whole bioethanol production process.

In summary, a computational scheme, that is practical and applicable, to integrate the experimental aspects of enzyme improvement with simulations of bioprocesses, where these enzymes are to be utilized, is lacking. This paper aims to present a workflow which demonstrates the abovementioned integrated approach on a case study. The workflow offers a dynamic link between experimentation and bioprocess simulation. Our research group has previously evaluated the white rot fungus *Pycnoporus sanguineus* DSMZ 3024 for the production of Lac, Bet, and Cmc as potential ingredients of a lignocellulolytic enzyme cocktail [22]. Also, a bioethanol production process utilizing hazelnut husk (HH) as a lignocellulosic feedstock and the abovementioned enzymes was conceptually designed, modelled, and simulated [23]. In this paper, a demonstration-of-concept level optimization of culture medium ingredients and culture duration for the production of the three enzymes using a process simulation-integrated scheme is presented.

2. Materials and methods

The main contribution of the present study to bioethanol process design and development literature is a methodological workflow which integrates early stage exploratory experiments for the optimization of lignocellulolytic enzyme mixture production with bioethanol production process simulations. The workflow is demonstrated on a case study.

The main carbon source in the enzyme production culture medium is HH. Addition of medium components (peptone as nitrogen source, yeast extract as additional carbon and nitrogen source, malt extract as additional carbon and nitrogen source, and sodium nitrate as an inorganic nitrogen source) and culture duration are optimized. A set of exploratory experiments are performed to collect enough data to establish three models to predict the activity of each enzyme under given culture conditions. A multi-objective Pareto optimization [24] of the three enzyme activities is performed to obtain a 21-sample non-dominated Pareto-optimal set of results. Each of the 21 results contains a set of culture condition values (auxiliary ingredient concentrations and culture duration) and accompanying enzyme activities that would be achieved under those conditions. None of the 21 results is superior to the others (it is a non-dominated set of results) meaning that no improvement can be achieved in any one of the three activities without compromising the others.

The critical step of the integrated optimization workflow is the estimation of a pseudo-enzyme cost (EC) based on culture medium composition and duration as well as a pseudo-enzyme cocktail performance metric (biomass conversion efficiency; CE) based on the achieved activities of the three enzymes. These two values are calculated for each of the 21 Pareto-optimal results and subsequently fed into the previously developed process model [23]. The overall process performance is simulated for each Pareto-optimal result. The point in the Pareto optimal set that gives the best process performance is selected as the overall optimal result. The optimization results are not only a function of culture conditions but also the bioethanol production process techno-economics. Process simulation-integrated optimization results are more informative compared to conventional standalone optimization results.

2.1. Experimental

2.1.1. Strain and chemicals

All of the chemicals and reagents used for experiments were purchased from Sigma unless stated otherwise. *P. sanguineus* DSMZ 3024 (Leibniz Institute-German Collection of Microorganisms and Cell Culture) was used as lignocellulosic enzyme producing microorganism. HH was supplied from local producers in northern region of Turkey. HH was prepared as described in [22] for further use as main carbon source.

2.1.2. Fungal growth and enzyme production

Malt extract agar (MEA) was used for fungal growth. MEA was prepared in 1 l with the following ingredients: 20 g malt extract, 5 g peptone from casein (peptone) and 20 g agar. Agar medium was sterilized at 121 °C, 1 atm for 15 min. Medium was cooled to 70 °C and agar plates were poured under laminar hood. Agar plates were inoculated with previously grown fungal cultures (cultures were passaged monthly and kept in 4 °C for further use). Agar plates were incubated at 37 °C for 3 days. Spore suspension was prepared as mentioned in an earlier study of our group [22].

Enzyme production medium contained 12 g/l HH and a total of 20 g/l additional nitrogen source as detailed in Table 1. pH was set to 7.0 with 1 M NaOH. HH was added as the sole carbon source to induce

Table 1
Enzyme production medium component ranges.

Medium Component	Concentration Levels (g/L)		
Peptone (A)	0	10	20
Yeast extract (B)	0	10	
Malt extract (C)	0	10	20
Sodium nitrate (D)	0	10	20

Independent variables A–D code for medium components in the designed experiments. Additionally, an independent variable E codes for sampling time points (E = 0–7).

lignocellulolytic enzyme production (Lac, Bet, and Cmc). Inoculation with spore suspension was done according to [22]. Production was carried out in 500 ml Erlenmeyer flask with 200 ml working volume. Time course of enzyme production was decided depending on the previous works of our group [22] and other groups [25,26]. Submerged production lasted for 7 days with daily sampling, at 30 °C and 180 rpm in a shaking incubator. All the experiments were done at least in duplicate.

Table 1 shows the concentration ranges of the medium components used in this study. The components and concentrations were selected as an interpretation of the authors' of the present study based on previous optimization studies on *P. sanguineus* [27] , *Pleurotus ostreatus* [28], and *Phlebia brevispora* [29]. The experiments performed were early exploratory investigations to understand the effect of nitrogen sources on the enzyme production. 6 exploratory experiments were designed as tabulated in Table 2.

2.1.3. Enzyme activity assays

2.1.3.1. Laccase. Lac activity was measured as described elsewhere [30] with following modifications: 15 mM ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt was used as substrate. 0.950 ml citrate buffer, 0.05 ml enzyme solution (if dilution was needed, final enzyme volume was kept as 0.050 ml), 0.2 ml ABTS (dissolved in 0.1 M sodium citrate buffer (pH 3.0)) solution were mixed in spectrophotometric cuvette at room temperature for 3 min and absorbance was read at 420 nm right after timeout. For blank solution, t = 0 sample was used as blank solution. One unit of Lac activity was expressed as the amount of enzyme required to oxidize 1 μmole of ABTS in a minute under the assay conditions.

2.1.3.2. β-glucosidase. Bet activity was measured as described elsewhere [31] with the following modifications: 10 mM pNPG (4-Nitrophenyl β-D-glucopyranoside) was used as substrate. 100 μl pNPG solution, 100 μl enzyme solution (if dilution was needed, final enzyme volume was kept 100 μl). 800 μl acetate buffer (0.1 M, pH 4.5) were mixed and the solution was put in water bath 45 °C for 15 min. After 15 min, 1 ml 1 M Na_2CO_3 was added to the tubes to stop reaction. Absorbance was read at 420 nm. One unit of Bet activity was expressed as the amount of enzyme required to release 1 μmole of pNP (p-Nitrophenol) per minute under the assay conditions.

2.1.3.3. Carboxymethyl cellulase. Cmc activity was measured as described elsewhere [32] with following modifications: 0.1 ml crude enzyme solution and 0.9 ml 2% carboxymetylcellulose (dissolved in 0.1 M sodium acetate buffer with pH 4.5) were vortexed in a heat resistant glass tube and incubated in 45 °C water bath for 5 min. 15 min later, 3 ml 3,5-dinitrosalicylic acid (DNS) was added, then tubes were incubated in boiling water for 10 min and transferred into ice. Absorbance was read at 540 nm after the sample cooled down to room temperature. One unit of Cmc activity was expressed as the amount of enzyme required to release 1 μmole of reducing sugar per minute under the assay conditions.

Table 2
Experimental design.

Experiments	A (g/L)	B (g/L)	C (g/L)	D (g/L)
1	20	0	0	0
2	10	10	0	0
3	0	10	10	0
4	0	0	20	0
5	0	10	0	10
6	0	0	0	20

2.2. Modeling and optimization

2.2.1. Enzyme activity models (Kriging)

A total of 48 activity data points were acquired for each of the three enzymes from time course experiments. Three empirical models were generated using Kriging [33] which is argued to be a better choice compared to more commonly used response surface models (low order polynomials) for highly nonlinear systems [34]. Our group has shown in a recent publication that Kriging outperforms conventional response surface methodology in the modeling of a complex biodiesel production scheme, using a limited number of exploratory experiments with predominantly temporal data [35].

Conventional response surface model structure is [36]:

$$y = f(x) + e \tag{1}$$

comprising a low order polynomial and a constant error whereas the Kriging predictor is [33]:

$$y = f(x) + z(x) \tag{2}$$

comprising a low order polynomial and a stochastic error.

Each model describes and can be used to predict the activity of one the three enzymes as a function of concentrations of medium components and culture duration. DACE Kriging toolbox [37] under MATLAB was used to generate the models.

$$Activity = f(A,B,C,D,E) \tag{3}$$

2.2.2. Multi-objective optimization (Pareto)

Since there are three enzymes of interest being produced, the culture conditions were optimized to obtain the best possible results for three activities; making this a multi-objective optimization problem with the following objective functions:

$$Activity_{Lac} = f(A, B, C, D, E) \tag{4}$$

$$Activity_{Bet} = f(A, B, C, D, E) \tag{5}$$

$$Activity_{Cmc} = f(A, B, C, D, E) \tag{6}$$

The multi-objective optimization problem was solved using the genetic algorithm-based multi-objective Pareto optimization functionality under MATLAB. The MATLAB function, gamultiobj (available under Global Optimization Toolbox), was executed with constraints on the independent variables. The objective functions used for Pareto optimization were the Kriging models generated for each enzyme (Eqs. (4)–(6)). The constraints for the independent variables were taken as the minimum and maximum values of the culture conditions used in the experimental design (Table 1).

The Pareto optimization procedure yields 21 results, each in the format of Eq. (7). Each result is a point in the multi-dimensional design space identified by a set of independent variable values (medium composition and culture duration) and the corresponding three enzyme activities that would be observed if enzyme production was performed under the identified conditions.

$$[Activity_{Lac_{Opt}}, Activity_{Bet_{Opt}}, Activity_{Cmc_{Opt}}]@f\left(A_{Opt}, B_{Opt}, C_{Opt}, D_{Opt}, E_{Opt}\right) \quad (7)$$

Each of these points lies on the non-dominated Pareto frontier. This means, for the three activity values at each point, that there can be no further improvements in any one of them without compromise in one or both of the others. In other words, each point on the Pareto frontier is equivalent in terms of multi-objective optimality.

2.2.3. Process simulation integration

Without the inclusion of another criterion, it is not possible to distinguish the best option out of the Pareto optimal results if optimization of enzyme production is performed independently. In order to allow a quantified determination of the best result along the Pareto frontier, an additional criterion is needed. The estimated cost of each individual result can be considered as an additional criterion. Although selecting the result with the lowest estimated cost would be an option, this could still produce a suboptimal situation when the performance of the whole bioethanol production process is considered.

Each Pareto optimal result has a different combination of media composition and culture duration which would generate a different cost. Also, each result has a different combination of enzyme activities which would generate a different performance in the bioethanol production process. Therefore, multi-objective Pareto optimization should be integrated with bioethanol production simulation in order to quantify the cost-performance relationship.

2.2.3.1. Bioethanol process model. In a previously proposed process model (hereafter called HH Process), HH was used as the lignocellulosic feedstock for a bioethanol production process [23]. A simplified process flow diagram is shown in Fig. 1. In the HH Process, HH was steam-pretreated in a reaction vessel and cooled down before lignocellulolytic enzyme mixture containing Lac, Bet and Cmc was fed into the vessel. Lac acted as a biological pretreatment agent as a secondary pretreatment of HH (after steam pretreatment), while Bet and Cmc catalyzed lignocellulose hydrolysis. The ligninolytic effect of laccase was shown previously by our group [22] and others [38,39]. Following the hydrolysis step, hydrolysate (as C source), peptone and yeast extract (as organic N sources) were fed into the fermentation vessel. Crude fermentation product (including bioethanol) was filtered through microfiltration and this filtrate was then distilled for final product recovery which was bioethanol with 95.5% alcohol content. All the design preferences, unit operation selections, and assumptions were taken from the referenced publication. The adopted model was used to run simulations without any changes from its original version, except enzyme cost and enzyme performance.

In the HH Process model, cost of enzyme mixture to hydrolyze 90% of the HH into fermentable sugars was assumed to be 2 $/kg. These values represent the best-case scenario since they are given as feasibility targets in [23]. In other words, the target for an enzyme improvement program would be to achieve the production of an enzyme mixture at 2 $/kg. The enzyme mixture would hydrolyze 90% of the HH.

2.2.3.2. Enzyme performance and cost integration. Optimization of enzyme production and the bioethanol production process are linked via the cost of the enzyme mixture (EC) and efficiency of the conversion achieved using the enzyme mixture (CE). The optimized conditions are assumed to dictate enzyme cost whereas conversion efficiency is taken as a function of the three activities achieved under the optimized conditions.

$$EC = f\left(A_{Opt}, B_{Opt}, C_{Opt}, D_{Opt}, E_{Opt}\right) \quad (8)$$

$$CE = f\left(Activity_{Lac_{Opt}}, Activity_{Bet_{Opt}}, Activity_{Cmc_{Opt}}\right) \quad (9)$$

90% conversion (value used in the bioethanol model) is assumed to be the best-case scenario when the maximum observed activities for each enzyme is considered (Lac_{max}, Bet_{max}, Cmc_{max}). So, optimization of enzyme production provides a possible reduction of the cost of enzyme from a maximum of 2 $/kg. The 2 $/kg cost is assumed to occur when maximum values of each operational condition is used e.g. A = 20, B = 10, C = 20, D = 20, and E = 7. Each of these factors are taken to affect the cost equally.

Each Pareto-optimal result involves optimal values for the operational conditions (A_{opt}, B_{opt}, C_{opt}, D_{opt}, E_{opt}) and accompanying activities achieved for the three enzymes (Lac_{opt}, Bet_{opt}, Cmc_{opt}). Because the production is a one-pot synthesis of the three enzymes together, it is not

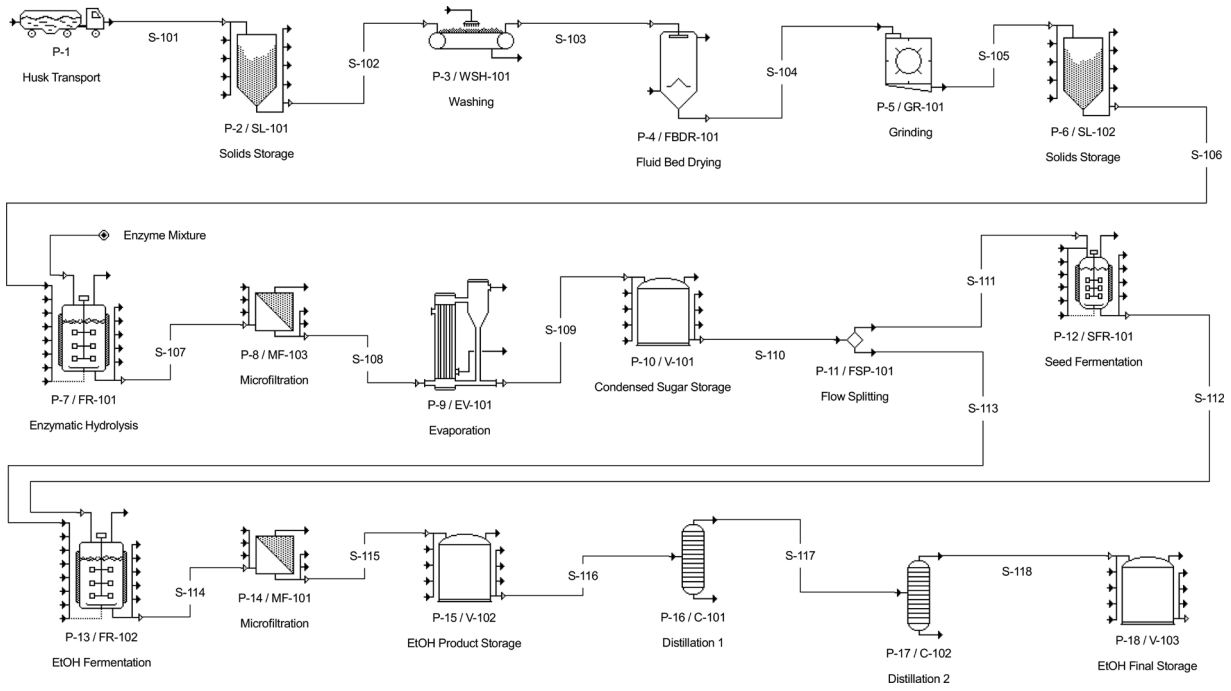

Fig. 1. Simplified PFD of HH Process.

possible to obtain optimal activities as high as those that could be obtained if conditions were optimized for each enzyme individually. How far the optimal activities of each enzyme are from their respective maximums is assumed to affect the overall conversion. Each enzyme is assumed to affect the overall conversion equally.

Following scheme is applied to calculate EC and CE:

A_{max} = 20; B_{max} = 10; C_{max} = 20; D_{max} = 20; E_{max} = 7 (maximum values from experimental design)

$Weight_A = Weight_B = Weight_C = Weight_D = Weight_E = 0.2$ (each condition has an equal effect)

$$coef_A = A_{opt}/A_{max} \quad (10)$$

$$coef_B = B_{opt}/B_{max} \quad (11)$$

$$coef_C = C_{opt}/C_{max} \quad (12)$$

$$coef_D = D_{opt}/D_{max} \quad (13)$$

$$coef_E = E_{opt}/E_{max} \quad (14)$$

$$EC = \$2/kg * (Weight_A*coef_A + Weight_B*coef_A + Weight_C*coef_C + Weight_D*coef_D + Weight_E*coef_E) \quad (15)$$

Lac_{max} = 581.7; Bet_{max} = 111.9; Cmc_{max} = 382.2 (maximum activity values obtained in experiments)$Weight_{Lac} = Weight_{Bet} = Weight_{Cmc}$ = 1/3 (each enzyme has an equal effect)

$$coef_{Lac} = Lac_{opt}/Lac_{max} \quad (16)$$

$$coef_{Bet} = Bet_{opt}/Bet_{max} \quad (17)$$

$$coef_{Cmc} = Cmc_{opt}/Cmc_{max} \quad (18)$$

$$CE = 90\% * (Weight_{Lac}*coef_{Lac} + Weight_{Bet} * coef_{Bet} + Weight_{Cmc}*coef_{Cmc}) \quad (19)$$

The calculated EC and CE were then fed into the bioethanol process model to calculate the net present value (NPV) of the process as described in [23].

3. Results and discussion

3.1. Enzyme activities

The activities of the three enzymes measured in the six exploratory experiments were tabulated in Table 3. Maximum activity of Lac (581.7 U/L) was observed in medium containing malt extract (ME) as additional nitrogen source. Maximum Cmc activity (382.2 U/L) was observed in medium containing peptone (P) as the sole nitrogen source and maximum Bet activity (111.9 U/L) was observed in the medium containing yeast extract (YE) and sodium nitrate (SN). The maximum activities of the three enzymes were all obtained the on 7th day of production. Arora and Sharma (2011) investigated the effect of nitrogen sources for bioprocessing of wheat straw in solid-state fermentation (SSF) via *P. brevispora* and reported that although digestibility increased with ME supplement, it also decreased the total organic content (TOC) by 18.8%. They concluded that increase in digestibility and decrease in TOC might be attributed to increase in biomass since ME is rich in simple sugar amino acids [29]. Moreover, Teoh et al. (2011) conducted a study to investigate the optimum medium composition for mycelial growth and antifungal activity of *P. sanguineus*. In this work, they found out that 10 g/l ME, YE, dextrose and maltose supported the highest fungal growth [27]. In the presence of readily available reducing sugar, fungi produce high levels of Lac enzyme [40]. In our experimental results, it is also seen that presence of ME led to increased levels of Lac activity, compared to other supplements. Increased biomass production could also be the reason of increased activity of Lac. Increased biomass production means increased enzyme activity due to cumulative effect of each cell's production.

Kachlishvili et al. (2006) stated that highest Cmc activity was observed with the medium containing beech tree supplemented with peptone during SSF via *P. dryinus* IBB 903, *P. tuberregium* IBB 624 and *L. edodes* IBB 363, which are members of Basidiomycota as *P. sanguineus* DSMZ 3024 [41]. In experiments containing ME, no significant change in Cmc activity was observed as time progressed. Since there is more than 60% maltose in ME [42], this medium is a suitable environment for the growth of *P. sanguineus* which was already grown on MEA for preparation of spore suspension. Therefore, the fungus had potentially readily expressed enzymes to degrade maltose. As fungal biomass increased, microbial degradation of HH increased too. This degradation could be leading to the extraction of phenolic compounds from HH as similarly observed by others [43].

Bet production media usually do not contain organic nitrogen source with some exceptions [44]. Most of the medium optimization studies for Bet production were done with the medium composed of lignocellulosic biomass impregnated with suitable salts (modified versions of minimal Czapek, Mandel and Reese medium etc) to induce Bet production. In our study, highest Bet activity was observed in Experiment 5, which contained SN as inorganic nitrogen source and YE as organic nitrogen source. When Experiments 5 and 6 were compared, it was obvious that Bet activities of Experiment 5 were higher than Experiment 6. During sterilization of the medium containing HH (which can be accepted as "partial" hydrothermal pretreatment of feedstock), phenolics and furan derivatives might have leaked to the medium. It was stated in [45,46] that hydrothermal pretreatment methods of lignocellulosic biomass release furan derivatives and phenolics. Phenolic acids, such as tannic and gallic acid inhibited the Bet activity in *Trichoderma reesei* [46]. Some studies in the literature state the possibility of Bet adsorption onto lignin [47] and inhibition of this adsorption is possible with addition of soybean protein [48]. YE might have caused a similar effect. Moreover, when all other experiments containing YE were analyzed, it was observed that presence of YE led to increased Bet activities in all cases (Table 3). Therefore, besides being a rich amino acid, vitamin and mineral source for microbial growth and maintenance of vitality, presence of YE might be presumed as a blocker for Bet adsorption onto lignin.

Table 3
Results of exploratory experiments.

Days	Experiment 1			Experiment 2			Experiment 3			Experiment 4			Experiment 5			Experiment 6		
	Lac	Bet	Cmc	Lac	Bet	Cmc	Lac	Bet	Cmc	Lac	Bet	Cmc	Lac	Bet	Cmc	Lac	Bet	Cmc
0	0.0	0.0	114.6	0.0	0.0	114.6	0.0	0.0	189.9	0.0	0.0	344.7	0.0	0.0	195.9	0.0	0.0	137.2
1	7.6	0.8	112.2	6.3	0.7	117.0	13.0	0.1	220.5	12.9	1.2	264.5	5.6	−13.2	194.8	2.8	−32.6	143.4
2	14.9	2.9	142.2	29.7	4.2	105.0	75.7	3.3	234.1	61.3	1.7	326.2	16.4	10.5	182.1	7.7	−40.7	134.1
3	24.6	17.8	139.0	59.1	36.0	127.4	131.7	14.4	243.9	92.3	18.3	296.4	28.9	35.6	186.4	12.4	−60.0	120.2
4	39.1	44.8	178.3	64.4	41.1	129.0	153.9	31.2	222.8	75.6	43.2	349.9	29.8	67.8	152.7	22.3	−52.9	131.8
5	35.5	28.9	237.5	50.6	51.6	144.6	54.3	42.9	312.3	111.1	7.0	168.8	32.9	86.1	183.8	29.9	−36.3	166.5
6	35.1	27.5	317.7	50.0	60.3	155.8	39.1	51.7	243.4	255.3	6.7	267.1	29.1	89.2	223.0	31.7	−32.2	131.0
7	69.0	30.4	382.2	48.9	52.9	159.4	102.1	49.8	365.8	581.7	26.2	276.3	30.1	111.9	242.3	32.9	−18.7	143.4

3.2. Activity models

Generally, exploratory experiments, such as those performed in this study, give preliminary insights into the nature of the new lignocellulolytic enzyme producing organism like those mentioned in the previous section. Subsequently, a new set of experiments were design based on the insights gained. The experimental design often follows a conventional statistical design of experiments approach such as response surface methodology. The primary objective of these experiments is the optimization of enzyme production. Usually they are static experiments with no temporal data. The data is then used to generate low order polynomial models of optimization objective functions (enzyme activities) which can be used in numerical optimization schemes.

Recently, it was shown that a different type of modeling approach, namely Kriging, allows the generation of models to be used in numerical optimization, using the time profile data from the exploratory experiments without the need to perform dedicated static experiments [35]. Accordingly, for this study, three Kriging-based models were generated for each enzyme activity as a function of the five enzyme production operating conditions. All three models showed good model statistics as tabulated in Table 4. The R^2 values indicate the percentage of experimental data variation explained by the models. Root mean square error values (RMSE) are a measure of the average deviations of model predictions from experimental values. The RMSE of Lac activity model was less than 1% of the maximum Lac activity observed. The RMSEs of Cmc and Bet activities were 5 and 7.7% of their respective maximums observed.

Fig. 2 shows the model predictions against experimental data, confirming the good fit between them. Lac activity model seems to be the best fitting with its respective experimental data. In comparison, Bet activity model shows very good fit for experiments 2, 3, 5, and 6 whereas the 4th day activity measurements that belong to experiments 1 and 4 show a slight mismatch. This may be due to a possible change in the behavior of the organism initiated on that day in the P and ME containing media. Whatever that change may be, the model seems to have incorporated it in the following data points for those two experiments. Cmc activity model arguably represents the overall trends in all of the experiments albeit not as accurately as the Lac and Bet activity models (as shown by the R^2 values). This can possibly be attributed to a higher number of uncontrolled factors affecting the Cmc activity assay compared to the Lac and Bet activity assays [43]. This is acceptable as long as a significant proportion of the overall experimental variation is explained by the model (R^2 = 0.85).

Table 4
Model statistics for the three activity models.

Activity Model	R^2	RMSE
Lac	0.997	4.7125
Bet	0.975	5.6449
Cmc	0.847	29.77

3.3. Optimization

The availability of activity models allows numerical optimization. The first part of the optimization workflow performs genetic algorithm based multi-objective optimization with the three activity models as objective functions. Five culture operating conditions (A–E) were optimized. 21 Pareto-optimal points were identified on the solution space. The resulting 3-D Pareto frontier is displayed in Fig. 3. Each Pareto-optimal point provided five enzyme production conditions and the corresponding enzyme activities that would be achieved, as predicted by the activity models. All Pareto-optimal points were equivalent. Trying to improve one of the activities would necessarily cause a deterioration in the others. Eight leftmost columns of Table 6 summarize the results of multi-objective optimization.

Process simulation integration of multi-objective optimization results was performed twice. In the first instance, the effects of five operating conditions on the enzyme cost and the three activities on enzyme efficiency were taken to be equal as detailed above. In the second instance, the effect of culture duration was considered to be more significant compared to the medium component concentrations. Also, Bet activity was considered to be more important compared to the activities of Lac and Cmc. The weight schemes for the two cases are given in Table 5.

The results of the optimization are tabulated in Table 6. In Case 1, each enzyme production operating condition was assumed to have an

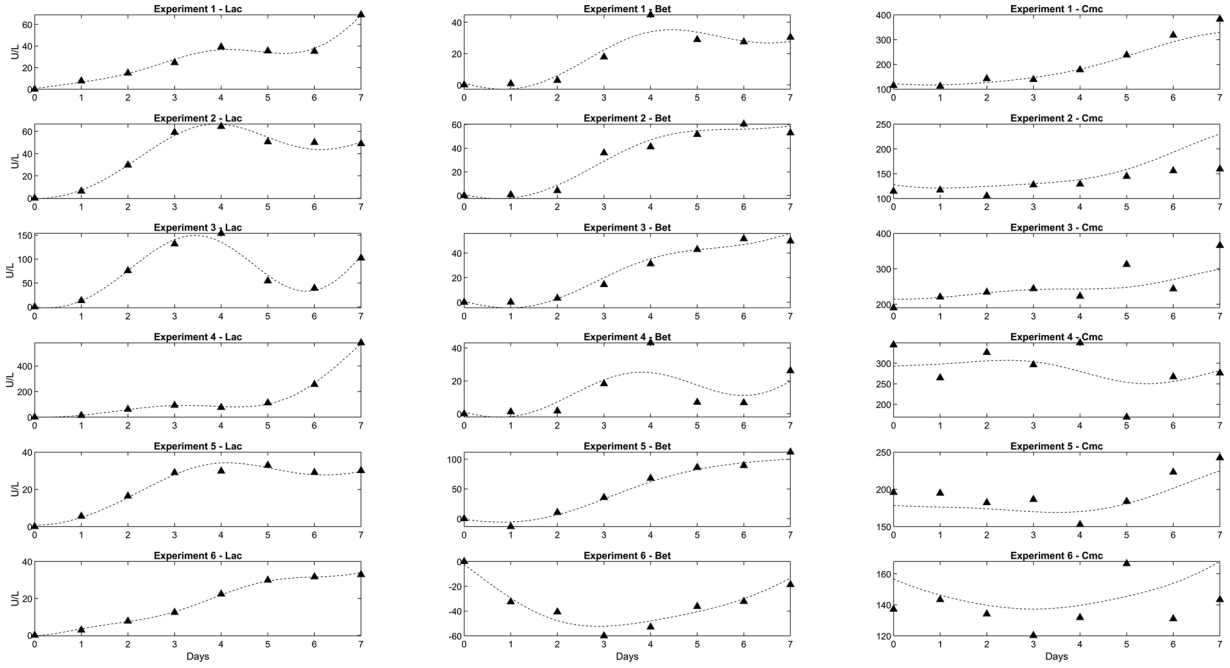

Fig. 2. Kriging model fit to experimental data.

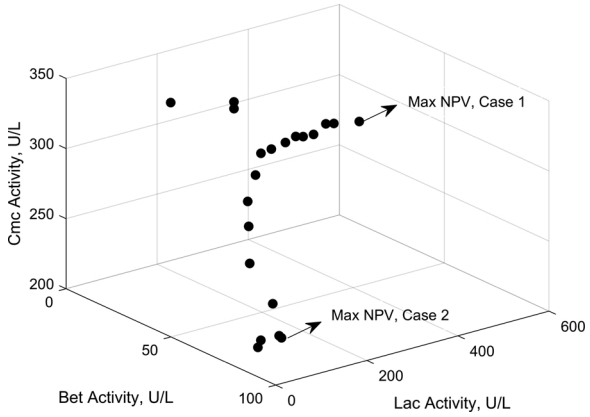

Fig. 3. 3-D Pareto frontier.

Table 5
Weight schemes for the two cases.

	Case 1	Case 2
$Weight_A$	0.2	0.1
$Weight_B$	0.2	0.1
$Weight_C$	0.2	0.1
$Weight_D$	0.2	0.1
$Weight_E$	0.2	0.6
$Weight_{Lac}$	1/3	0.5/3
$Weight_{Bet}$	1/3	2/3
$Weight_{Cmc}$	1/3	0.5/3

equal effect on the overall cost of enzyme production. This is a simplifying assumption to demonstrate the application of the workflow. In a different enzyme production system, the effects of different operating conditions on the cost of enzyme will vary. In Case 1, the Pareto-optimal result with the best simulated NPV is detailed in the fourth row of Table 6. NPV is simulated to be $76.1 million. The enzyme production operating conditions for this result require minimum P (1.1 g/L), YE (0.2 g/L) and SN (0.4 g/L) use. ME is required near its maximum level (19.8 g/L). Culture duration required was seven days.

In Case 2, culture duration is considered to be a more significant factor contributing to the cost of enzyme production. This is reasonable because a long culture requires an increased input of utilities and consumables while at the same time lowering productivity. Also, in Case 2, Bet activity is considered to have a more significant effect on the performance of the lignocellulolytic enzyme cocktail. Bet is usually the limiting enzyme in most lignocellulolytic enzyme mixtures [49–52], making this assumption a reasonable case to investigate. When necessary modifications were made to the weighing scheme to reflect the considerations of Case 2 (as detailed in Table 5), the Pareto-optimal result with the best simulated NPV changed, compared to Case 1. As in Case 1, minimum P and SN and maximum culture duration were required. However, in contrast to Case 1, Case 2 required maximal YE and minimal ME. In Case 2, simulated NPV was increased to $106.6 million.

The response surfaces for Cases 1 and 2 relating the combined effects of yeast and malt extract concentrations in the enzyme mixture production on NPV of bioethanol production process are given in Fig. 4. Response surfaces for other combinations of culture ingredients can be found in Supplementary Materials.

4. Conclusion

We have developed and demonstrated a process simulation-integrated multi-objective optimization workflow for lignocellulolytic enzymes production. Time-profile data from six experiments with varying medium compositions were collected within an exploratory experimentation scheme. The data were used to generate enzyme activity models by Kriging for three lignocellulolytic enzymes, Lac, Bet, and Cmc, as a function of medium composition and culture duration. We had previously shown that Kriging performs better compared to more conventional response surface methodology with temporal data sets from similar exploratory experiments. The generated models were used to perform multi-objective optimization of medium composition and culture duration using a Pareto-based approach. The Pareto-optimal results were linked via enzyme performance and enzyme cost functions to a bioethanol production process simulation which had been developed by our group before. The cases, where significance of enzyme production conditions affected bioethanol production differently, were analyzed with the process-simulation integrated

Table 6
Process simulation-integrated multi-objective optimization results.

Pareto Optimal Operating Conditions for Enzyme Production					Model Predicted Enzyme Activities			Calculated CE (%)		Calculated EC ($/kg)		Simulated NPV ($ mil)	
Peptone (g/L)	Yeast Extract (g/L)	Malt Extract (g/L)	Sodium Nitrate (g/L)	Duration (Days)	Lac (U/L)	Bet (U/L)	Cmc (U/L)	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2
20.0	0.5	6.9	0.6	7	143.7	18.7	333.0	38.6	26.8	0.97	1.48	−38.1	−148.0
1.1	9.9	0.3	7.4	7	32.0	95.8	228.0	45.2	61.2	0.97	1.46	8.5	106.6
5.3	9.7	0.6	10.5	7	48.3	81.0	209.6	40.7	52.9	1.11	1.52	−20.8	56.0
1.5	0.2	19.8	0.4	7	559.5	18.3	282.7	56.0	35.3	0.84	1.42	76.1	−66.8
2.1	7.6	19.5	2.9	7	363.4	30.6	297.7	50.3	37.5	1.19	1.60	40.7	−49.3
1.8	7.1	17.3	3.0	7	325.1	34.0	299.1	49.4	38.4	1.13	1.56	33.1	−41.5
0.2	9.7	0.7	7.6	7	29.1	95.3	229.4	45.1	60.9	0.94	1.43	7.4	104.9
0.4	9.6	2.9	7.5	7	43.2	89.2	246.8	45.5	58.6	1.00	1.50	9.7	91.1
1.2	9.0	6.3	6.8	7	72.7	71.8	261.0	43.5	50.6	1.03	1.49	−2.2	42.2
1.1	6.0	7.3	5.9	7	142.3	56.2	270.6	43.7	44.4	0.93	1.46	−1.0	2.7
17.2	0.5	15.9	0.7	7	322.6	10.0	311.8	43.8	25.9	1.09	1.53	−0.5	−156.8
1.1	0.7	16.1	2.2	7	444.4	21.5	285.8	51.1	34.2	0.81	1.40	46.3	−76.7
2.0	8.5	15.3	2.1	7	247.9	39.1	301.7	47.0	39.2	1.13	1.56	18.4	−34.3
1.7	7.4	12.3	3.3	7	207.4	45.3	294.0	45.9	41.2	1.04	1.51	12.2	−17.4
2.1	7.9	16.2	2.7	7	280.4	37.0	300.4	48.0	38.8	1.14	1.57	24.5	−37.5
4.1	0.4	19.3	0.2	7	505.5	14.1	282.9	52.1	31.7	0.88	1.42	52.0	−99.8
4.8	9.7	0.7	8.3	7	45.6	83.0	216.3	41.6	54.2	1.05	1.49	−13.7	63.9
1.3	7.9	11.7	6.2	7	165.0	50.8	282.7	44.3	42.6	1.09	1.53	2.6	−8.6
1.8	4.6	17.3	3.0	7	388.8	28.6	294.0	50.8	36.9	1.03	1.51	44.0	−53.7
19.7	0.5	17.7	3.2	7	321.3	10.3	307.3	43.5	25.9	1.23	1.60	−2.9	−157.7
1.5	0.2	17.3	0.4	7	498.7	19.4	287.4	53.5	53.5	0.79	1.39	60.9	−73.7

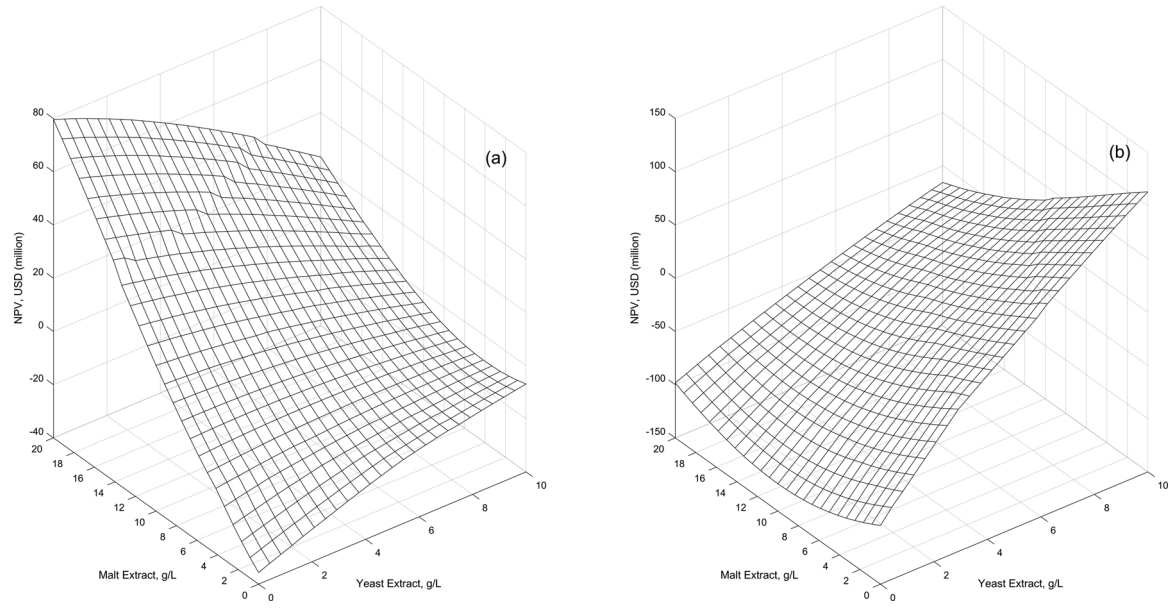

Fig. 4. Combined effect of YE and ME concentrations in enzyme production on the NPV of bioethanol production process; a) Case 1 b) Case 2.

optimization workflow. A shift from a ME to YE-dominated medium requirement was observed in Case 2 compared to Case 1. This observation was made possible with the process simulation-integrated optimization workflow.

This demonstration hopefully highlights the importance of process simulation integration from the earliest stages of biocatalyst screening, selection, and optimization not only for lignocellulolytic enzyme production towards bioethanol manufacturing but also for the wider biocatalysis field. The present manuscript was intended as a demonstration of the concept. Many details entail further refinement not the least of which regards enzyme performance and cost calculations and related weighing schemes. This is the subject of ongoing studies by our group.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2019.107420.

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