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Enhancement of xylanase productivity using industrial by-product under solid suspended fermentation in a stirred tank bioreactor with dissolved oxygen constant control strategy

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Abstract

Solid suspended fermentation of *Bacillus subtilis*D3d using different agro-industrial residues were performed in this work to co-production of xylanase and lichenase. Based on the results, brewer's spent grain (BSG) and corn steep liquor (CSL) combination were selected for further studies. The central composite design with the aim of maximum enzymes production at minimum fermentation time indicated 2.5% BSG and 0.5% CSL at 42 h is the best condition to highest productivity in shake flask. The results revealed that CSL at high level can lead to reduction of enzyme productivity. In a 2 L stirred tank bioreactor the productivity of xylanase reached a peak of 10 U/(ml.h) using dissolved oxygen (DO)-constant control strategy at 50% air saturation. Lichenase production increased 1.7 times but the productivity of this enzyme was not affected by DO levels. The physiological state of solid suspended fermentation was investigated by carbon dioxide evolution rate (CER) and oxygen transfer rate (OTR) through off gas analyzer data.

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1. Introduction

In recent years, there is great interest to reduce the pollution arising from agricultural and industrial activities. Most industries are trying to modify their processes to reuse of residues or finding new applications of agro-industrial waste as raw material[1].

Lignocellulosic materials (LCM), mainly from agriculture residues, forestry sources, industrial by-products[2]can be used as substrate in fermentation to produce value added products such as lignocellulolytic enzyme[3]that is prerequisite to establish green environmental technology and bio refinery. Fermentable sugars are the main products of LCM hydrolysis and can be fermented to ethanol[4], butanol[5], xylitol[6] . Recently the production of oligosaccharides such as xylooligosaccharides[7] the value added products of partial hydrolysis of LCM have also been increasingly studied[8].

In addition to reduction production cost, using LCMs as substrate can induce lignocellulolytic enzyme systems, hydrolytic (glucanases and xylanases) and ligninolytic (laccase and lignin peroxidise) enzymes. Consequently, the co-production of lignocellulosic enzymes eliminates the cost of separately enzyme preparation and blending that is carried out to improving the lignocellulolytic enzyme bio catalytic effects via synergistic actions.

LCM consists mainly of cellulose, hemicellulose, and lignin. The most abundant hemicelluloses are comprised of xylans, that frequently consist of heterogeneous mixtures of pentoses (xylose, arbinose), hexoses (mannose, glucose, galactose) and sugar acids[9]. The some LCMs that are rich in hemicellulose include corncobs, sugarcane

bagasse, rice husks, barley straw, olive pits, wheat straw and bran, cotton, tobacco and sunflower stalk and brewer's spent grain[10-12]. Hydrolysis of xylan is catalysed by Xylanases and a mixture of shorter xylo-oligosaccharides, xylobiose and xylose is produced. Xylanases have been reported mainly from bacteria [13, 14], fungi [15, 16] and actinomycetes[17].

β-Glucans are the major cell wall components in cereals. The degradation of β-glucans in nature is catalysed by β -glucanases, which, based on the type of glycosidic linkage that they cleave, can be grouped into four main categories, namely 1,3;1,4-glucanases (lichenases, EC 3.2.1.73), 1,4-glucanases (cellulases, EC 3.2.1.4), 1,3-glucanases (laminarinases, EC 3.2.1.39), and 1,3(4)-glucanases (EC 3.2.1.6)[18].

Xylanases and glucanases have attracted much attention because of their applications in industrial processes such as the modification of cereal-based food stuffs, improving the digestibility of animal feed stocks, fruit softening and clarifying juices, improvement of bakery products, textile industry, pre-bleaching of paper pulps and so on[19, 20].

For the economic enzyme production, it is necessary to identify microorganisms that produce high levels of enzymes using inexpensive and easily available substrates such as LCMs and other by-products as carbon and nitrogen sources. As LCMs are found in the form of agricultural and industrial residues, they could be utilized as potential substrate for growing the microorganisms and bio molecules production. Optimization of culture medium and conditions can significantly enhance the enzyme production. Enzyme production is a multiple variables biochemical reaction and the response surface methodology (RSM) is a commonly used method to assess the optimal fermentation conditions[21] and also an efficient statistical technique for estimation of

variables and their interaction significance with a low number of experiments, saving time and costs. Many researchers have confirmed this method improves enzyme production by submerged and solid state fermentation[22].

This work reports optimization of xylanase and lichenase co-production medium with agro-industrial residues as substrate by *Bacillus subtilis*D3d. Following statistical optimization, the effect of LCM concentration on the harvesting time was investigated by the RSM results. The aim of this study is to develop an economical culture medium using agro-industrial by-products. Regarding the lack of solid state fermentation industrial development, to take advantage of the agricultural residues in industrial scale to produce value added products out of solid wastes, it seems the engineering aspects of solid suspended fermentation of LCM with high concentration must be further developed in stirred bioreactors which are currently widely used in the industry. For example, in this way, to optimize energy consumption in such systems, oxygen uptake rate (OUR) and oxygen transport rate (OTR) should be taking into special consideration, hence parameters such as viscosity changes during fermentation due to the growth of microorganisms, protein excretion and substrate hydrolysis. To increase enzyme production and productivity, we did further experiment using optimized medium in stirred tank bioreactor and batch culture. The effect of dissolved oxygen (DO) concentration on the productivity of both bacterial xylanase and lichenase was investigated. In addition, direct biomass estimation in solid state or suspended fermentations is usually impossible, because the efficient separation of biomass from the substrate matrix is impractical. Therefore in this work, the growth was indirectly traced by measuring of the CO_2 evolution rate (CER) and OTR through an off-gas analyser CO_2/O_2.

2. Materials and Methods

2.1. Chemicals and raw materials material

Beechwood xylan and lichenan were purchased from Sigma-Aldrich Co. Wheat bran, soybean and canola meal was supplied from local markets. Other agro-industrial residues were obtained as following: BSG from the Behnoosh Brewery Co. in Tehran, CSL as well as corn germ from Glucusan Co. in Ghazvin, sugarcane bagasse and vinasse from Razi Yeast and Alcohol Co. in Abadan, and tomato seed from Golgashte Shirin plant CO. in Ghazvin, Iran.

2.2. Bacterial strain and inoculum preparation

*Bacillus subtilis*D3d was isolated from apricot phyllosphere collected from Kerman, Iran. This strain was identified by the Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Iran. Stock cultures are being maintained on nutrient agar at 4 °C. Seed culture of *B.subtilis*D3d was prepared in nutrient broth at 30°C for 18h.

2.3. Enzyme production

Xylanase production in SmF was carried out in the basal medium consisted of the following (in g/L) yeast extract, 3; meat extract, 2; K_2HPO_4, 1 and $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.5, beechwood xylan as carbon source and xylanase inducer, 10. The cultivation was carried out in a 250 ml flask containing 50 ml culture media on a shaking incubator (160 rpm) at 30 °C. After 48h of incubation, the contents of the fermented flasks were centrifuged at 10000g for 20min at 4°C and the supernatants were used for enzyme activity.

2.4. Enzyme assay

The xylanase activity was determined according to [23] and the released reducing sugars from beechwood xylanase substrate (1%, w/v in 100mMTris/HCl; pH 7.0)at 50°C for 20 min, were measured using the 3,5-dinitrosalicylicacid(DNS)[24]. Briefly, 1ml reaction mixture containing 40µl of appropriately diluted crude enzyme in same buffer and 360µl of the substrate. The reactions were stopped by adding 600µl of DNS reagent followed by heating the reaction mixtures in a boiling water bath for 15 min and cooling the mixture by immersing in ice water bath. The concentration of reducing sugar was measured by absorbance at 540 nm using D-xylose as a standard. The blank and control mixture contained 360µl of a 1% (w/v) substrate and 40µlinactivated diluted crude enzyme in same buffer, respectively. The diluted crude enzyme was inactivated by immersing in ice water bath prior to adding DNS. One unit of xylanase activity is defined as the amount of enzyme that releases 1µmol of xylose equivalent reducing sugar per minute under assay conditions. In addition, lichenase with lichenan as substrate (0.5% in 100mM citrate buffer; pH6) at 50°C for 30 min. Reducing sugar release from substrate were determined using DNS method and comparing to glucose as standard. All experiments were carried out in triplicates and the presented results are mean values ± SD.

2.5. Xylanase and lichenase co- production using agro-industrial by-product

2.5.1. Carbon source

Brewer's spent grain (BSG), Wheat bran (WB) and sugarcane bagasse (SCB) as carbon sources were tested each at a concentration of 1.0% (w/v). Enzymes co-production was done further by 3 and 5% BSG. These experiments were performed in three replications (three Erlenmeyer flasks).

2.5.2. Nitrogen source

Various industrial by products were used as nitrogen source at 0.5% concentration (w/v). Vinasse, CSL, corn germ, soy meal, canola meal and tomato seed were investigated in our research.

2.5.3. Optimization of xylanase and lichenase co-production by *B.subtilis*D3d

The RSM was used to optimize the co-production of xylanase and lichenase in the submerged cultivation system, which was supplemented with concentration of BSG, CSL and the incubation time were chosen as the independent variables, and the enzyme activity(U/ml) was used as the dependent variable. The influence of variables was investigated in the 1-5% BSG, 0.5-3% CSL and 24-56h for time cultivation range via a central composite design (CCD) on 5 levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) with $\alpha = 1.68$, conform presented at Table.1.The experimental ranges were defined based on initial studies. The quadratic polynomial model (Eq. (1)), was calculated to estimate the response of the dependent variable for the production of xylanase and lichenase at each condition.

$$Y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 \quad (1)$$

where Y is predicted response (enzyme activity), A, B, and C represent concentration(%) of BSG and CSL and incubation time, respectively. b_0 is the offset term, b_1, b_2 and b_3 are linear effects, b_{12}, b_{13}, b_{23} are interaction terms and b_{11}, b_{22}, and b_{33} are squared effects. The experimental design and the regression analysis of the data were done using the Design expert statistical package (Stat-Ease Inc., Minneapolis, MN, USA). The variables, levels, experimental design of the CCD and the actual

responses are shown in Table.1. The value of the dependent responses was the mean of three replications.

2.6. Solid-suspended fermentation in a stirred tank bioreactor

The fermentations were conducted in a New Brunswick 2L stirred tank reactor (BioFlo/CelliGen310). The production medium was inoculated with 5% v/v of 18-h-old seed culture from nutrient broth. In all experiments, the temperature was kept at 30°C and a pH of (6.8-7.2) was maintained using automatic additions of NaOH (20%) and H_2SO_4 (2%).Dissolved oxygen (DO) was measured with a membrane-covered palaeographic oxygen electrode which was heat-sterilizable (Metteler Toledo, MA, USA). Here, three levels of dissolved oxygen (DO 30, 40 and 50%) were used to investigate the effect of DO on xylanase and lichenase production. The DO concentration was maintained at defined level, with aeration varying between 0.5 and 3vvm, and stirring speeds were between 250 and 900 rpm. The foam was controlled by addition of refined canola oil. The cultivations were continued up to 30h, and samples were removed every 4h. The sample was centrifuged (12,000×g, 15 min) and the supernatant being used for measurement of enzyme activity.

2.6.1. Respirometry study

Because of the solid substrate presence, we could not use optical density or dry mass methods to measure cell growth. Therefore, we used Off O_2/CO_2 gas analyser (New Brunswick EX-2000) for bacterial growth tracing.

The OTR can be determined from gas oxygen mass balance on the bioreactor as follows:

$$OTR = \frac{Q}{V}\left(C_{O2}^{in} - C_{O2}^{out}\right) \quad (2)$$

where Q is the oxygen gas flow, V the volume of liquid, and C^{in} and C^{out} the oxygen concentration measured at bioreactor inlet and outlet, and well-mixing of the gas phase has been assumed[25].

Carbon dioxide evolution rate (CER) can be calculated from Eq.(3)

$$CER = \frac{Q}{V}(C_{CO2}^{out} - C_{CO2}^{in}) \quad (3)$$

with applying ideal gas law the OTR and CER presented in g/l dimension. Schematic diagram of enzyme production in stirred tank bioreactor equipped with off-gas analyzer is shown in Fig. 1. The total reducing sugars concentration measured using DNS method.

3. Results and discussion

3.1. The effect of carbon sources on the enzyme production

One of the parameters that must be optimized in microbial fermentation medium is carbon source that has main role in overall cellular growth and metabolism. Hydrolysing LCM enzymes can be produced by commercial pure substrate, also agro and industrial residue. From the great majority of the reports on microbial production of cellulases and hemicellulases it is shown that the LCMs have the greatest impact on lignocellulolytic enzymes secretion. Certain substrates may significantly stimulate lignocellulolytic enzyme synthesis without supplementation of the culture medium with pure inducers[26]. In this research we tried to use brewers spent grain (BSG, main solid by-product of the beer production process (brewing)), wheat bran (WB) and sugarcane bagasse(SCB) to investigate their effect on xylanase and lichenase co-production. Given that our first priority is increasing xylanase production by *B.subtilis*D3d, we chose

mentioned residues because the hemicellulose content of them is high and approximately close together, 20-40%[26], 35.5-39.5%[27], 28-32% [28, 29], respectively for BSG,WB and SCB. Although one of the factors that would effects enzyme production using LCMs substrate is availability of xylan that is different in various LCMs. In the first stage, enzyme production was carried out by3% of each substrate concentration. Table.2 shows highest enzyme production was obtained with BSG. Therefore we investigate enzyme production at 1, 3 and 5% of BSG. We found that 3 and 5% BSG have the almost identical enzyme production after 48h. However, we could not test further BSG in flask because of insufficient mixing. Mittal et al reported the xylanase production was found to vary with changes in the wheat bran concentration. The highest enzyme activity was found at 2 % (w/v), and there was a decline in xylanase production when the concentration of wheat bran was increased beyond 2 %[30].Due to the formation of a dense suspension of the substrates and insufficient mixing in shake flasks the solid substrate concentration is restricted.

3.2. The effect of Nitrogen source on the enzyme production

Enzyme production was measured in the presence of several residues as nitrogen sources using 1.0% (w/v) BSG as selected carbon source. In most researches, the nitrogen source selected between organic and inorganic materials such as urea, yeast, meat and beef extract, peptone, casein hydrolysate[17], KNO_3, NH_4NO_3 and $NaNO_3$ both individually and in combination. However, organic nitrogen sources have been more enhancing effect on xylanase production. At this work, for reduction of production cost we used some industrial by products as nitrogen source, corn steep liquor, vinasse, corn germ, soybean and canola meal, and also tomato seed. The results of this investigation

were illustrated in Fig.2. from environmental point of view, investigation into using industrial by-products in microorganisms cultivation and bio-component production could be resulted to find the alternatives for some by-product with the more environmental contamination effects like as vinasse[31].In this research we have not conducted any treatment on industrial by-products and only the possibility of enzyme production was investigated. However in some case such as vinasse, corn and canola meal the xylanase production diminished. According to Fig.2, *B.subtilis*D3d produces approximately 11U/ml lichenase from 1% BSG that was similar in all cases although corn meal induced lichenase production up to 13.5U/ml. It revealed that corn germ is a good candidate for lichenase production. Soybean meal, CSL and tomato seed have enhanced xylanase production. However, soybean meal is the most important protein source used in feed animals and therefore its price is higher in comparison with the cheap substrate such as CSL. The xylanase production in the presence of soybean meal and CSL was approximately similar and because we would to optimize the medium composition for submerged fermentation and for sufficient mixing, we prefer using CSL as nitrogen source. Also, based on our knowledge the composition of BSG and tomato seed to xylanase production has not been reported earlier. Table.3 summarises the most recent attempts on lignocellulolytic enzyme enzymes production using some agricultural or industrial by-products.

3.3. Optimization of enzyme production by RSM

In this research base on concentration of BSG (carbon source), CSL (nitrogen source) and cultivation time as independent variables 20 experiments was designed with CCD to optimize xylanase and lichenase co-production. Table4 shows the variables, levels

and experimental conditions. In order to investigate the effect of each factor on the responses, analysis of variance (ANOVA) results were calculated and the statistical significance of the models equation was evaluated by the *F*-test which showed that the regression was statistically significant. The ANOVA tables are available in the supplementary data. The 'Prob>*F*' value for the both of models was <0.0001, which indicated that the models were statistically significant with a confidence interval of 99.99 %. The coefficient of determination (R^2) of the model was 0.9686 and 0.9711 for xylanase and lichenase models respectively, which shows that the models were suitable for adequately representing the real relationships among the selected reaction variables. Xylanase and lichenase activity (U/ml) were fitted with a second-order polynomial equation (Eqs. (4 and5)) in terms of coded factors:

Xylanase activity = $+86.10 - 3.19\,A - 3.82\,B + 18.55C + 3.38AB + 10.50AC + 1.82BC - 10.64\,A^2 + 0.21B^2 - 6.98C^2$ (4)

Lichenase activity = $+4.51 - 0.56A - 2.01B + 3.96C - 0.60AB - 0.30AC - 0.82BC + 0.59A^2 + 1.57B^2 + 1.26\,C^2$ (5)

where independent factors A(%) , B(%) and C(h) denote BSG, CSL and time, respectively.

Fig. 3 shows the predicted data versus actual data obtained from the experimental results. The clustering of the points around the diagonal line indicates a satisfactory correlation between the experimental data and the predicted values, confirming the validity of the model. The relatively high R^2 and adjusted R^2values indicate that the quadratic model is capable of representing xylanase and lichenase production under the given experimental conditions.

3.3.1. Response plots

Fig. 4a and b shows the two dimensional response plots of the relationship between BSG and CSL concentration at the central value of incubation time (40 h) and BSG and incubation time at central value of CSL (1.75%) on xylanase production. As the contour plots depict, response interaction of CSL along with BSG suggested that a decrease in the concentration of CSL leads to an increase in xylanase activity. Furthermore, Fig3b shows at a constant level of CSL, an increase in the time of incubation enhances xylanase activity. And, this effect is particularly evident in higher concentrations of BSG that more time is needed to obtain maximum enzyme production.

These plots, the p-value in analysis of variances and the coefficient of time terms in equations 4 and 5 demonstrate the importance of the time as a factor in the medium optimization with lignocellulosic carbon sources. It seems that different initial concentrations of simple sugars in medium containing varying amount of the LCM can have a direct impact on biomass growth and consequently maximum enzyme production time and productivity.

In the case of lichenase production as is shown in Fig. 5, similarly, the lower level of CSL is preferred and, the effect of BSG on lichenase production is lower than xylanase. Although CSL is very rich in essential nutrients but higher concentrations of CSL have negative effect on enzyme productivity due to adding sugar content to medium or presence of inhibitory components like of lactic acid[32]. Wang et al. investigated the effect of phenolic compounds in CSL on laccase production and reported sinapinic acid in CSL caused a reduction in laccase production[33].Using CCD with substrate concentration and incubation time as independent parameters, the production and productivity could be optimized with less experiments and more precision.

3.3.2. Confirmatory tests

Table 4 displays the composition of medium to achieve higher productivity. To test the validity of the model, three experiments were carried out under mentioned optimal conditions. The results of the confirmation experiments indicate that actual responses at optimum condition and the predicted values from the fitted correlations were in close agreement at a 95% confidence interval. These results satisfactorily confirmed the validity of the model.

After optimization of the BSG and CSL with the aim of maximum enzyme productivity, the enzymes production investigated at the controlled conditions in a stirred tank bioreactor.

3.4. Xylanase and lichenase co-production in solid-suspended batch culture

LCMs hydrolytic enzymes can be produced by solid state fermentation (SSF) or submerged fermentation (SmF) in shake flask or in bioreactors. Nowadays the SmFs are the most common of commercial bioprocesses since it provides better monitoring facilities and ease of handling. Nevertheless, the solid state fermentations (SSFs) due to the significant reduction of production cost by decreasing substrate and downstream processes are recently considered. The solid suspended fermentation enables a reduction in the cost of medium components and at the same time provides better control on process.

Using solid agro-industrial by-products as carbon or nitrogen substrate requires development of solid-suspended fermentation. These low cost and high nutritive substrates enhance induction of different enzymes and also would reduce the catabolic repression due to high concentration of fermentable sugars.

Given the low solubility of oxygen in aqueous solutions, DO in the broth can be the limiting substrate[25]so it is an important influencing factor in the process of aerobic microbial fermentation. A relatively high DO in the fermentation process would lead to high energy consumption; contrariwise, relatively low DO would negatively affect cell growth. The effects of dissolved oxygen (DO) tension on the xylanase or cellulase production in fungi have been studied by researchers[34]. Recent study reported by Zhang et al investigates the influence of DO concentrations on biomass and enzyme production. The cellulase and xylanase production by *Streptomyces griseorubens* JSD-1 reached a peak at DO 50%[35]. In most cases of enzyme production mixing speed and aeration rate were varied in order to keep DO values above the critical level[36]. However, the effect of DO concentration on the productivity of both bacterial xylanase and lichenase has not been adequately studied.

To investigate the effects of DO on enzyme production, three oxygen levels were studied at constant DO (30%, 40% and 50%). Any other fermentation conditions were not changed in these processes. The trends of effect of DO concentration on xylanase and lichenase production are observed Fig. 6a and b, respectively.Fig.6a shows although maximum enzyme production has not been increased significantly by higher amount of DO, the xylanase productivity enhanced. The graph reveals that there has been a sharp rise in xylanase activity after hour 10 at different DO levels. But, at hour 13, highest xylanase productivity of 10U/(ml.h) was achieved at 50% air saturation.The productivity at DO 40% and 30% reached to 7.5 and 6 U/(ml.h) at the same time. Increasing in DO level probably positively affect metabolic activity during exponential and stationary growth phase in a way that maximum xylanase production achieved in a shorter time.

The trend of lichenase production differs from xylanase production. As shown in Fig. 6b lichenase activity increased during 5-25h of fermentation time with almost constant slope and after hour 25 probably remained steady. The results show that lichenase production is not significantly changed by dissolved oxygen concentration at the studied range.

The total reducing sugars concentration profiles during cultivations under controlled conditions in bioreactor are shown in Fig7. It can be seen that there was a decrease in the reducing sugars concentration at the beginning of the cultivation, and xylanase production started after reduction in reducing sugars. It seems that after exhaustion of initial simple sugars, xylanase production induced by xylooligosaccharides[37]. After rapid enzyme production, the enzyme activity and reducing sugars concentration remains almost constant. This was probably related to the equilibrium between sugars consumption and production due to enzymatic hydrolysis and then reducing sugars accumulation was seen illustrating the hydrolysis rate is higher than consumption rate at the end of cultivation.

Fig. 8 projects the OTR and CER profiles over fermentation time. According to the CER profile, Lag phase lasted for about an hour. This phase represented the adaptation to the culture medium hence the amount of CO_2 produced (and diluted by the air flow) was almost nil. CER and OTR exponentially increase after lag-phase. After 5h, CER and OTR level off until hour 6, and rise exponentially in parallel until hour 8. Two peaks on OTR profile would be represented diauxic growth[38] on BSG as substrate. Thereafter, the OTR and CER decrease indicated the end of exponential phase. A comparison of Fig 5 and 7 indicates lichenase production have been induced after first exponential phase and increased with almost constant slope. While, xylanase production

increased after second exponential phase. Pereira et al. produced thermo stable cellulase-free xylanase by *B. subtilis* and reported xylanase production is associated with cell growth[13],However, Schneider et al. described xylanase production by a *B. subtilis* strain after growth phase[37]. It appears that the composition of medium, initial reducing sugars concentration, cultivation condition and other produced enzymes during the fermentation have the significant role in growth associated or non-associated of enzyme production.

4. Conclusion

Based on our knowledge, this is the first report about the combination of BSG and CSL to co-production of bacterial xylanase and lichenase. Statistical multi-objective optimization of enzyme production was successfully carried out using RSM based on CCD. The results revealed that CSL at high level can lead to reduction of enzyme productivity. 2.5%BSG, 0.5%CSL and 42h is the best condition to the highest productivity of xylanase and lichenase. Enzyme production at bioreactor enhanced 1.7 times for shaker flask for both enzymes. Xylanase productivity remarkably improved with dissolved oxygen concentration at 50% air saturation. Nevertheless, lichenase production is not significantly changed by dissolved oxygen concentration at the studied range. CER and OTR trends indicated off gas analysis and respirometry studies would be provided useful information of solid suspended fermentation. Further studies on enzyme production and process evaluation and optimization to scale up solid suspended fermentation are underway.

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Tables and figures captions

Table 1: The variables and their levels, the CCD and actual responses

Table 2: Enzyme production with different carbon sources

Table3: Recent applications of agricultural and industrial by-products as substrate for xylanase and lichenase production

Table 4: Numerical optimization with maximizing enzyme activity, minimizing time and verification of the model at optimum condition

Fig. 1: Schematic diagram of the batch culture system for enzyme production equipped with off-gas analyzer

Fig. 2: Enzyme production with 1% BSG and 0.5% different by product as nitrogen source and control without nitrogen source.

Fig. 3: The observed production of (a): xylanase and (b): lichenase versus the predicted production under the experimental conditions.

Fig. 4: Contour plot of the (a): BSG and CSL effect at 40h cultivation time and (b): the BSG and incubation time effect at CSL 1.75%on xylanase production

Fig. 5: Contour plot of the BSG and CSL effect on lichenase production at 40h cultivation time.

Fig. 6: Time course of (b): xylanase and (b): lichenase production by *B. subtilis*D3d at different DO level.

Fig. 7: Total reducing sugars concentration during co production of xylanase and lichenase by *B. subtilis*D3d at different DO level.

Fig. 8: OTR and CER trends during solid suspended fermentation of *B. subtilis*D3d at DO50%

Table 1: The variables, levels, the CCD and actual responses.

		Coded levels				
Variable	symbol	-α	-1	0	+1	+α
BSG%	A	1	1.80	3	4.20	5
CSL%	B	0.50	1	1.75	2.50	3
Time(h)	C	24	30.50	40	49.50	56

	Factors			Actual Responses U/ml	
Run	BSG%	CSL%	Time(h)	Xylanase	Lichenase
1	3.00	1.75	56.00	96.09	13.77
2	5.00	1.75	40.00	47.22	4.67
3	3.00	3.00	40.00	80.76	4.72
4	3.00	1.75	40.00	84.00	4.69
5	3.00	1.75	40.00	83.00	4.49
6	1.80	2.50	30.50	57.27	2.93
7	4.20	2.50	49.50	96.49	7.85
8	1.00	1.75	40.00	57.72	7.33
9	3.00	0.50	40.00	85.57	12.85
10	4.20	1.01	49.50	95.75	15.43
11	4.20	1.01	30.50	45.77	4.52
12	3.00	1.75	40.00	90.84	5.77
13	1.80	1.00	49.50	89.35	14.35
14	4.20	2.50	30.50	35.19	2.80
15	1.80	2.50	49.50	73.83	11.73
16	3.00	1.75	40.00	91.00	4.00
17	3.00	1.75	40.00	85.00	4.00
18	3.00	1.75	40.00	84.00	4.18
19	3.00	1.75	24.00	29.49	2.04
20	1.80	1.00	30.50	77.30	4.82

Table 2: Enzyme production with different carbon source*

	Xylanase (U/ml)	Lichenase (U/ml)
Brewers' spent grain (BSG)	106.54±1.84	3.94±0.05
Wheat Bran (WB)	100.35±1.45	3.20±0.06
Sugarcane Bagasse (SCB)	37.54±0.65	1.40±0.08

*3% (w/v) carbon source, yeast extract 3 g/l, meat extract 2 g/l

Table 3: Recent applications of agricultural and industrial by-products as substrate for lignocellulolytic enzyme production

Microorganism	Enzyme	Substrate	Ref
*Bacillus subtilis*D3d	Xylanase and lichenase	BSG and CSL	This work
Streptomyces griseorubens JSD-1	cellulase and xylanase	rice straw, wheat bran	[24]
Bacillus pumilus	xylanase	Pulp and Paper Sludges	[25]
Coprinuscomatus with *Trichodermareesei*	CMCase, xylanase and laccase	corn cob, corn stover wheat bran	[3]
Bacillus sp. UEB-S	lichenase	millet	[39]
Bacillus subtilis	xylanase	Soybean hull, wheat bran and pineapple peel	[40]
Sporotrichum thermophile	xylanase	De-oiled Jatrophacurcas seed cake	[41]

Table 4: Numerical optimization with maximizing enzyme activity, minimizing time and verification of the model at optimum condition

response (U/ml)	BSG (%)	CSL (%)	Time (h)	Predicted response	Actual response	95% CI
Xylanase and Lichenase	2.5	0.5	42	97.00 and 13.46	94.33±3.45 and 12.62±0.85	86.46-106.54 and 11.61-15.62
Xylanase	2.2	0.5	33	84.30	82.56±2.8	72.65-95.95
Lichenase	5	0.5	24	10.17	11.26±0.74	5.27-15.07

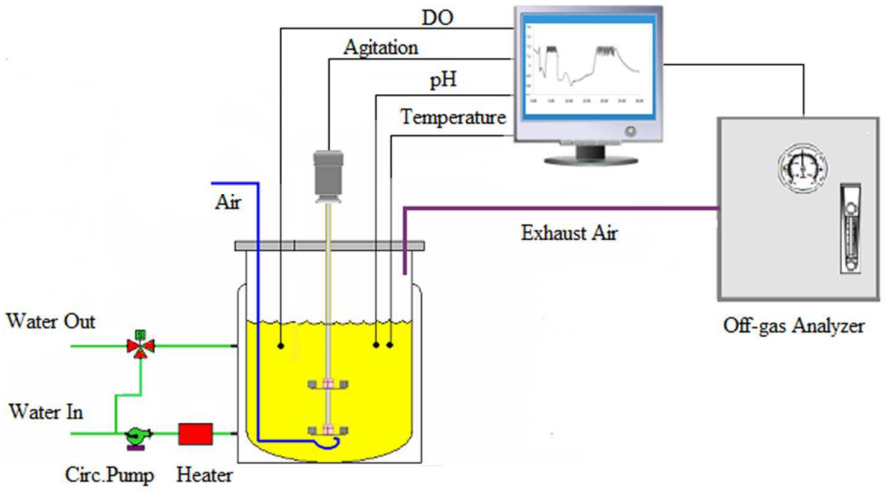


Fig. 1

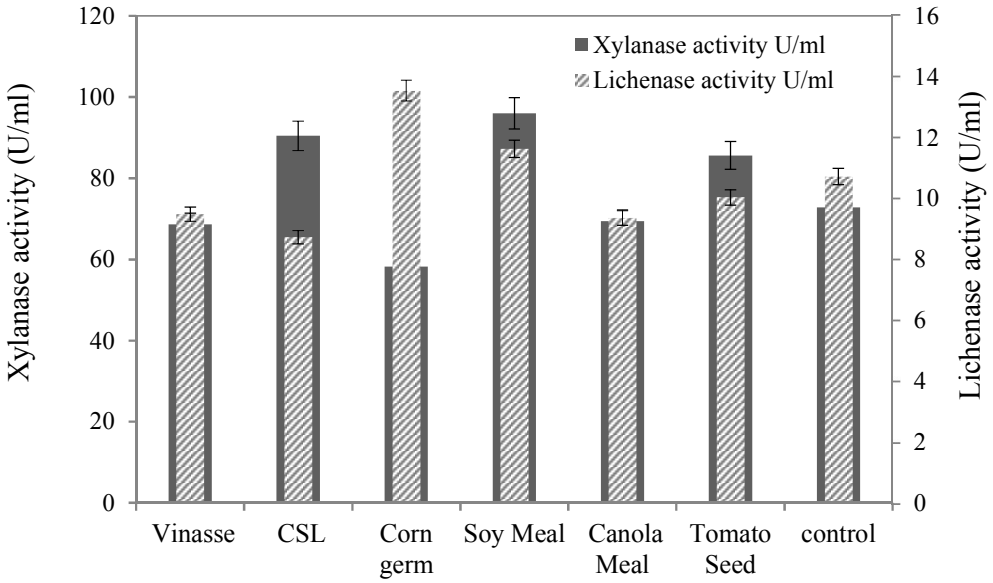
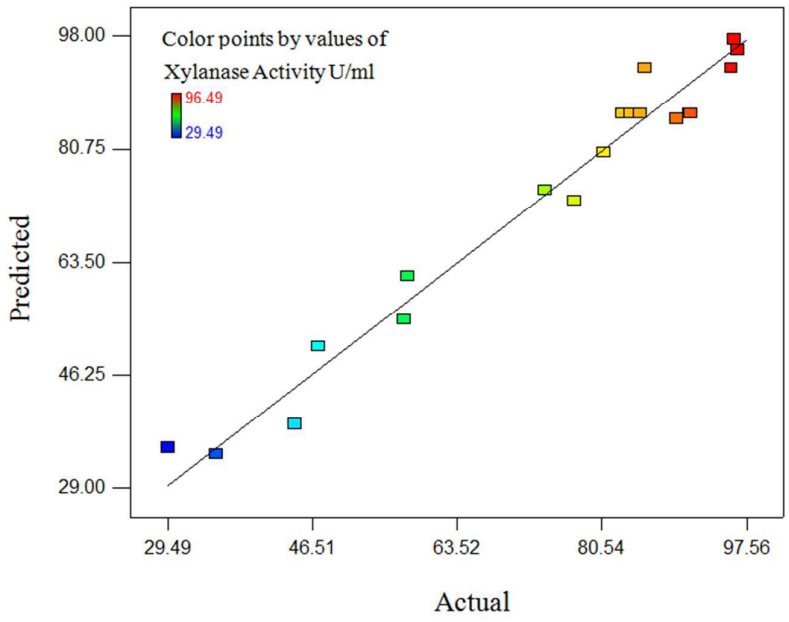


Fig. 2

(a)



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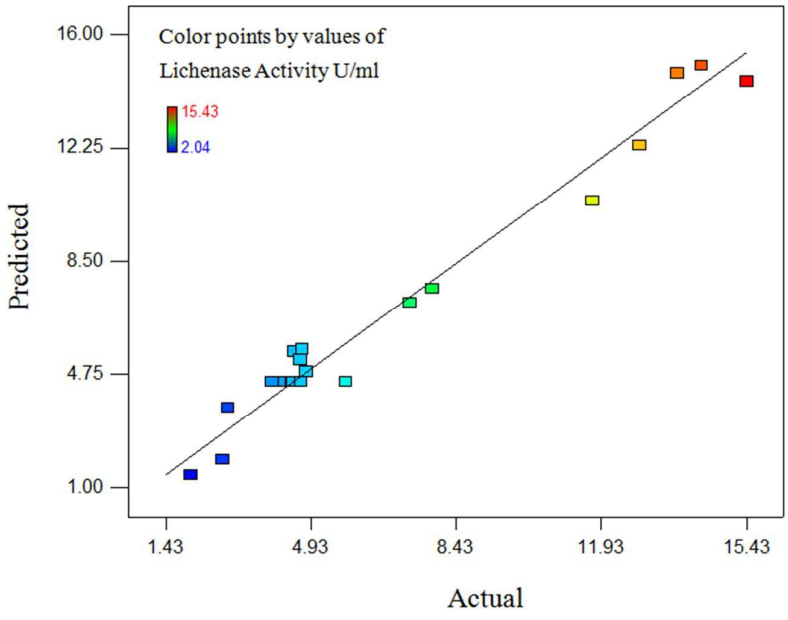
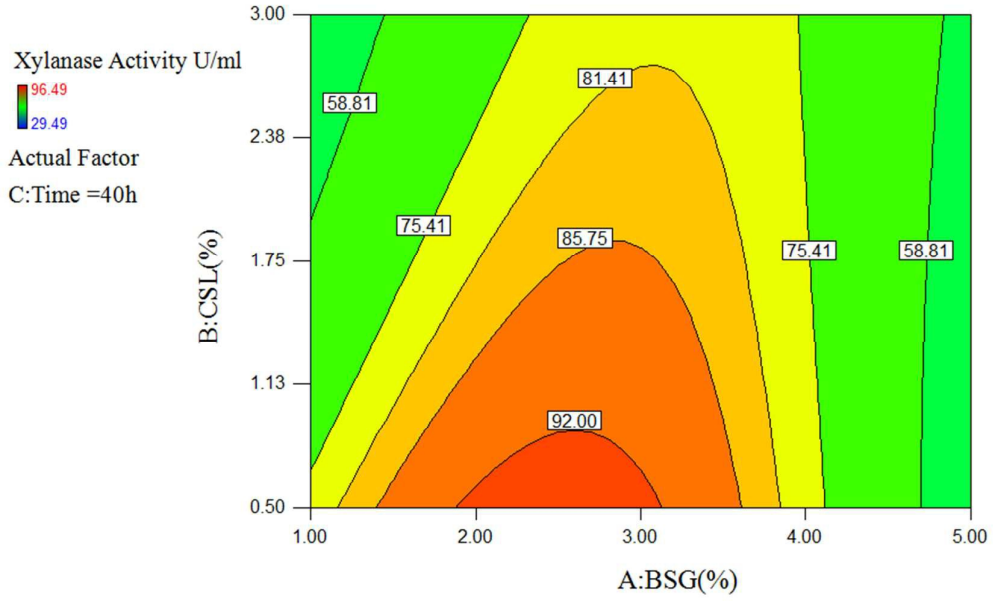


Fig. 3

(a)



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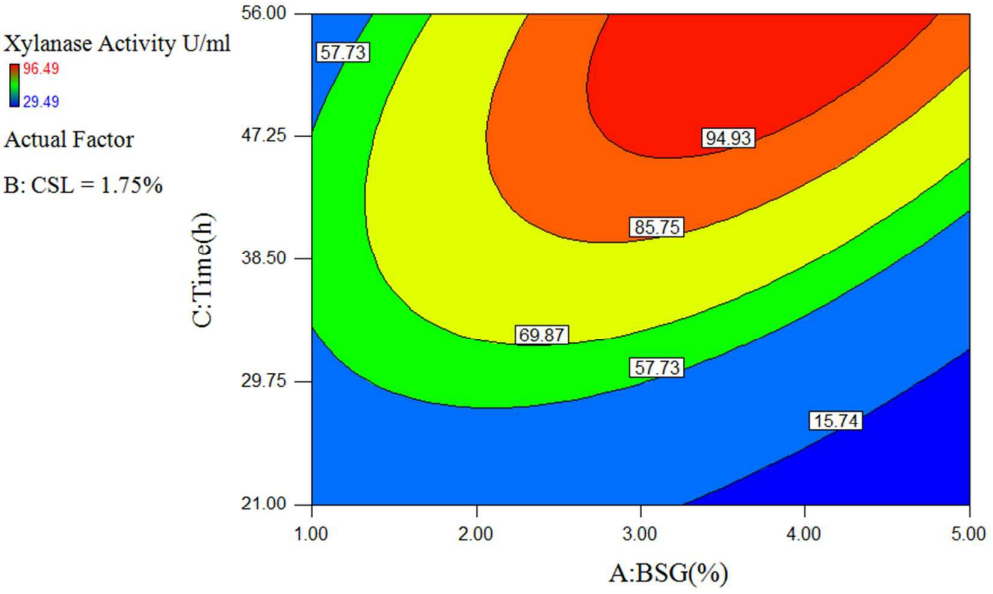

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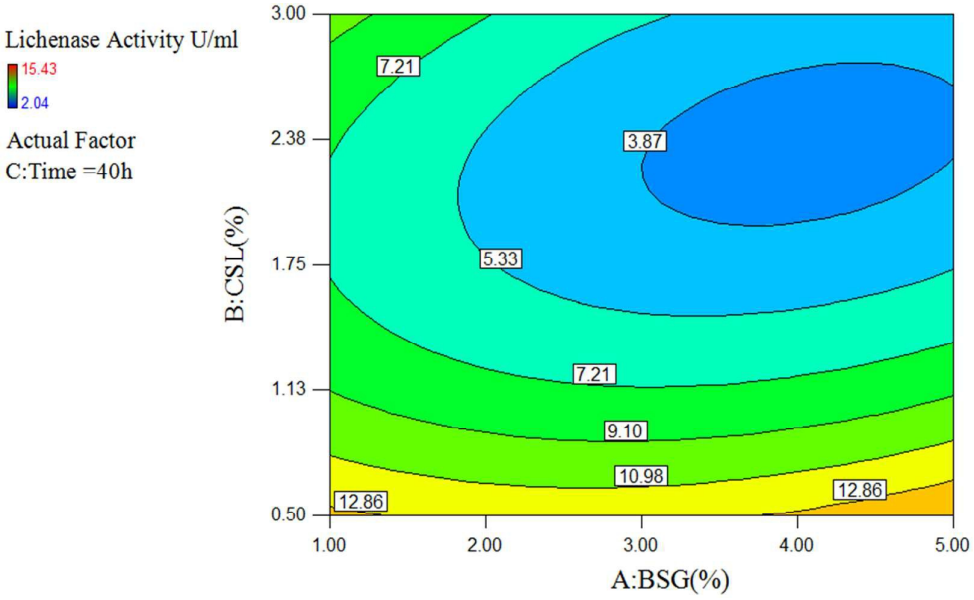
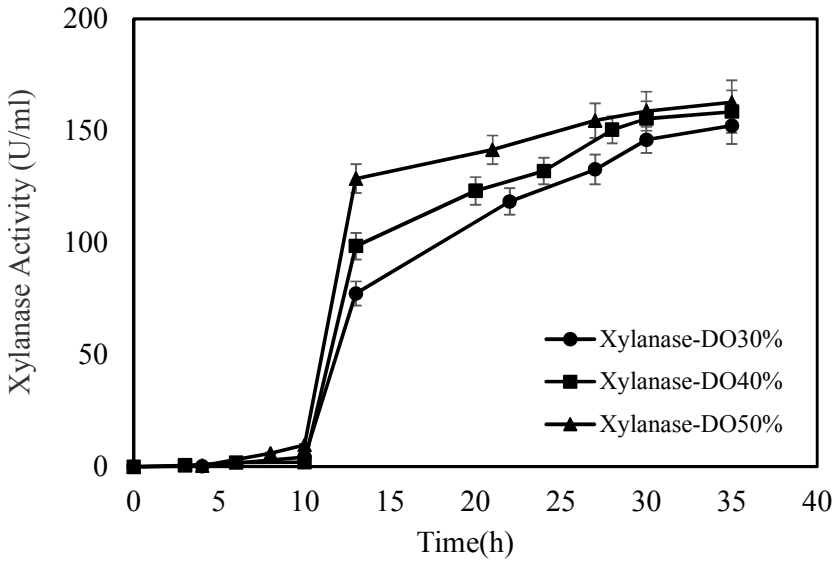


Fig. 5

(a)



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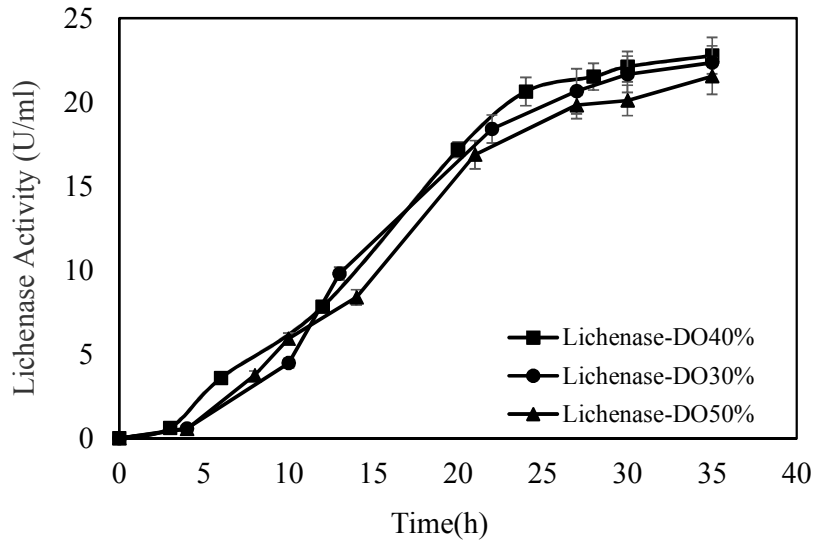

Fig. 6

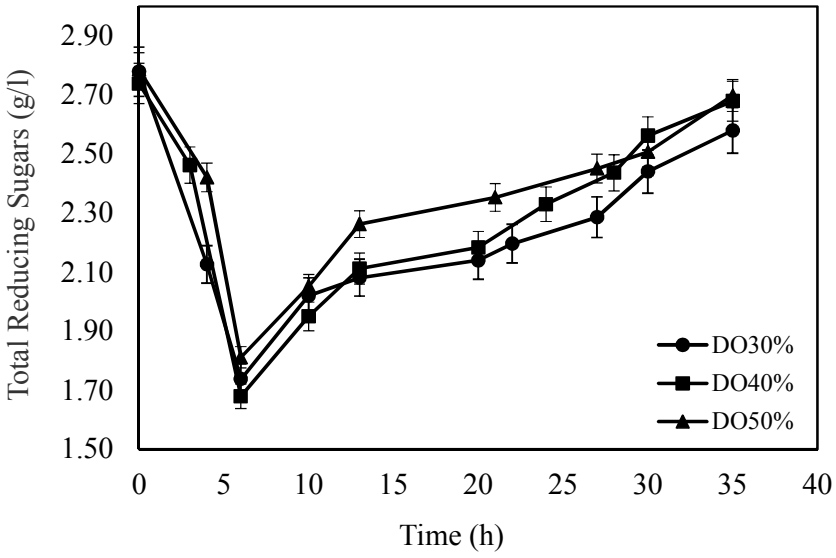

Fig. 7

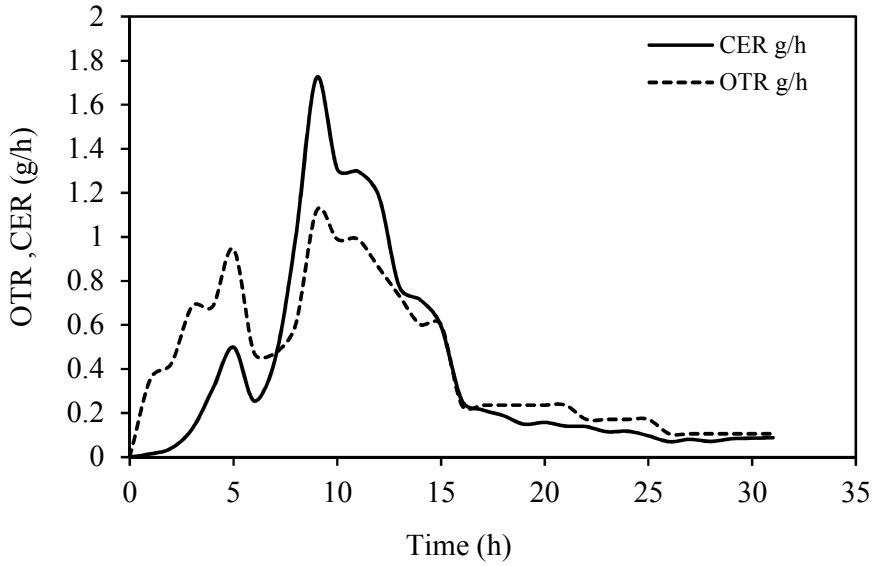

Fig. 8