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Feasibility of Sago bioethanol liquid waste as a feedstock for laccase production in recombinant *Pichia pastoris*

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Abstract

The use of waste stream generated upon the production of bioethanol from sago fibre has not been a major focus in the literature thus far. This study explores the feasibility of utilising the liquid waste generated following the production of bioethanol from sago fibre as a feedstock for the production of recombinant laccase in Pichia pastoris GS115. Characterisation of the sago bioethanol liquid waste (SBLW) indicated that glycerol was the main constituent along with glucose and lactic acid. Our results showed that P. pastoris GS115 growth was generally feasible when SBLW was used as a feedstock irrespective of its concentration.

The expression of laccase reached the highest in fermentations that employed 40% (v/v) SBLW, whilst higher concentrations of SBLW resulted in the reduction of the laccase activity. The highest laccase activity achieved using 40% (v/v) SBLW represented 73% of that obtained using the standard synthetic medium. Further enhancement of maximum biomass concentration by 1.2-fold and laccase titre by 1.5-fold was achieved when the 40% (v/v) SBLW was supplemented with 1.0% (w/v) yeast extract. This work gives useful insights into the promising applications of SBLW as an inexpensive and sustainable feedstock for the production of industrial biocatalysts.

Keywords: Laccase, biocatalyst, bioethanol spent broth, sago, sago fibre, *Pichia pastoris*, valorisation.

Introduction

Exploitation of agricultural waste as alternative feedstocks for bioethanol production has gained increasing interests among biorefineries. One of the potential agricultural wastes in Malaysia is sago fibre, which composed of approximately 50% to 60% residual starch along with other lignocellulosic components[2]. The use of hydrolysate of sago fibre as a substrate for producing bioethanol has been reported by Awang-Adeni and co-workers[2].

Given a far-sighted context, with the increasing demand for bioethanol at the industrial level in the future, it is estimated that the production of the waste stream following bioethanol distillation will also increase. Direct disposal of the waste stream may lead to serious environmental pollutions due to the high organic content, dissolved solids and other toxic compounds[9]. Therefore, there is an emerging need to investigate the fate and potential of the waste stream generated following the bioethanol production.

Research in Brazil, India and Europe has shown the feasibility of exploiting vinasse, the glycerol-based stillage discharged after the industrial production of bioethanol from molasses derived either from sugary crops (beet and sugarcane), starchy crops (wheat and cassava) or cellulosic materials[6], as feedstocks for the production of an array of value-added products such as polyhydroxyalkanoates[5,15] and xylitol[17]. Similar potential could also be envisaged in the case of the stillage generated from the bioethanol production using sago fibre as a substrate. To date, the exploitation of the waste stream generated following the production of bioethanol from sago fibre still remain unexplored. Thus, a fundamental investigation into properties and applications of the waste stream will shed some light on its rational fate and direction.

One of the industrially relevant enzymes, laccases, was used as the model case throughout this study. Laccases are polyphenol oxidases that have important applications in the treatment of effluents from paper and pulp, textile and petrochemical industries[7], bioremediation of xenobiotics and as biosensors in medical diagnostics[8]. Previously, a laccase producing strain, *Pichia pastoris* GS115 was established by Sing[18]. Nonetheless, development of strategies for optimal and inexpensive production of laccases by *P. pastoris* GS115 is still under-examined since the strain is relatively new. Preliminary studies of laccase production by *P. pastoris* GS115 were based on a synthetic medium, Buffered Methanol-Complex Medium (BMMH), which is fossil-based and often deemed as expensive. Hence, there is a need for developing a cost-effective and sustainable strategy for producing laccases in *P. pastoris* GS115.

The main aim of this study was to evaluate the feasibility of sago bioethanol liquid waste (SBLW) as a fermentation feedstock for the production of laccases by *P. pastoris* GS115. The work entailed characterisation of SBLW prior to its evaluation for *P. pastoris* GS115 fermentation and laccase production. Following the establishment of the optimal strategy for using SBLW as a fermentation feedstock, the work focused on the enhancement of laccase production by supplementing the diluted SBLW with yeast extract. The findings of this study will provide an early insight into the potential use of SBLW for growing *P. pastoris*, which is one of the widely used hosts for producing recombinant enzymes. The utility of SBLW could be further extended to other bioproductions producing various target proteins.

Material and Methods

Microorganisms: *Saccharomyces cerevisiae* was used for the bioethanol fermentation in this study. *P. pastoris* GS115 containing pPICZB/eLcc1 vector that incorporates the laccase gene from *Marasmius cladophyllus*, developed from a preceding study[18], was used in this work. Both strains were obtained from Molecular Genetics Laboratory and Biochemistry Laboratory of Faculty of Resource Science and Technology, UNIMAS respectively.

Media: Sago fibre collected from Herdson Sago Mill, Pusa, Saratok, Sarawak, was used as the feedstock for bioethanol production. Pre-treatment and hydrolysis of sago fibre were based on a method by Awg-Adeni et al.[1] The resulting sago fibre hydrolysate (SFH) was used as a substrate for bioethanol fermentation by *S. cerevisiae*. Buffered Methanol Glycerol Histidine (BMGH), which comprised of 0.1 M potassium phosphate, 0.02% (w/v) biotin (ACROS, China), 13.4% (w/v) yeast nitrogen base (YNB) (BD Difco, France) and 0.4% (w/v) histidine (Sigma-Aldrich, USA) was used as a medium for seed culture.

BMMH, which was used as a standard medium for laccase production, comprised of 0.1 M potassium phosphate, 1.34% (w/v) YNB, 0.02% (w/v) biotin, 0.004% (w/v) histidine and 0.5 % (v/v) methanol (Merck, Germany). The initial pH of BMGH and BMMH was adjusted to pH 6 prior to the sterilisation. Distilled water was used in all preparative procedures.

Bioethanol fermentation by *S. cerevisiae*: Bioethanol fermentation by *S. cerevisiae* was performed in 250-mL shake flasks with a working volume of 100 mL. SFH with the initial concentration of glucose of 55 g L^{-1} was used as a substrate in all fermentations. The cultures were incubated at 30 °C on an orbital shaker (Gyromax™ 706 Hotech Instruments Corp., Taiwan) at 150 rpm for 48 hours. Aliquots of the culture broth were withdrawn every 12 hours in order to quantify the cell growth and bioethanol. The spent broth was harvested after 48 hours. Upon the bioethanol fermentation, the culture broth was centrifuged at 10 000 rpm for 10 minutes. The pellet was discarded whilst the resulting supernatant (SBLW) was recovered and heated at 80 °C in order to remove bioethanol.

SBLW was analysed for its composition using High Performance Liquid Chromatography (HPLC) before it was autoclaved and prepared for use as a feedstock for *P. pastoris* GS115 fermentation. SBLW was prepared at different concentrations: 40%, 50%, 60% and 100% (v/v) where BMMH was used as the solvent.

***Pichia pastoris* GS115 fermentation:** The seed culture was initiated by transferring a single colony of *P. pastoris* GS115 into 40 mL of BMGH medium in 100-mL baffled shake flask. The seed culture was then incubated overnight at 30 °C on an orbital incubator shaker at 170 rpm. Once the absorbance of the seed culture reached between 5 and 6, the culture was then withdrawn and centrifuged at 4000 rpm for 10 minutes before it was transferred to fresh BMMH (control) or SBLW for *P. pastoris* GS115 fermentation.

The seed density was standardised to 10% (v/v). The cultures were incubated at 30 °C on an orbital shaker at a shaking speed of 170 rpm. For every 24 hours, 0.5% (v/v) of absolute methanol was added to the cultures in order to induce the expression of recombinant laccases. During the fermentations, aliquots of culture broth were withdrawn for every 48 hours. The cell growth was monitored via absorbance measurement. Another portion of sampled broth was centrifuged at 4000 rpm for 10 minutes and the resulting supernatant was used for laccase assay.

Analytical methods

Cell growth analysis: The absorbance measurement of *S. cerevisiae* and *P. pastoris* GS115 culture was carried out at 575 and 600 nm respectively using a spectrophotometer. Whenever needed, the sampled broth was diluted with distilled water such that the absorbance readings remained below 0.9. The absorbance values were translated into equivalent dry cell weight (DCW) based on an established standard curve.

Laccase assay: The activity of laccase was determined based on the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)[18]. The reaction mixture consisted of 0.4 mL culture supernatant consisting of laccase, 0.1 mL 5 mM ABTS and 0.5 mL of 0.1 M sodium acetate buffer (pH 5). Next, the reaction mixture was incubated for 2 minutes at room temperature.

Following that, the absorbance value of the mixture was measured using a spectrophotometer at 420 nm. The blank was prepared using boiled culture supernatant. The laccase activity was calculated using equation 1:

$$Enzyme\ activity\ \left(\frac{U}{mL}\right) = \frac{\Delta A \times V \times 10^3}{v \times \varepsilon \times d} \quad (1)$$

where ΔA represents absorbance change (minute^{-1}), V is total reaction volume (mL), v is enzymatic extract volume (mL), ε is molar absorptivity (36000 M^{-1} cm^{-1}) and d is cuvette diameter (cm). One unit of activity (U) was defined as the amount of laccases to oxidise 1 µmol of ABTS per minute.

High Performance Liquid Chromatography (HPLC): Bioethanol, glycerol, glucose and lactic acid were quantified using HPLC (Shimadzu, Kyoto, Japan). The system was fitted with an Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Co., USA) with the injection volume of 20 µL, column oven temperature of 65 °C monitored with a refractive index detector. The column was eluted with 0.4 mM sulphuric acid as the mobile phase at a flow rate of 0.4 mL min^{-1} at 30 °C. Retention times for glucose, lactic acid, glycerol and bioethanol were 4.1, 5.5, 5.8 and 8.9 min respectively.

Statistical analysis: The experimental data were statistically analysed using Minitab 17 Software (Minitab® 17.1.0, USA). The Tukey's Test was used to compare the means via one-way analysis of variance (ANOVA).

Results and Discussion

Characterisation of sago bioethanol liquid waste (SBLW): SBLW was generated following the bioethanol fermentation by *S. cerevisiae* using SFH as a substrate. Fig. 1 shows the cell growth and bioethanol profile during *S. cerevisiae* fermentation. The maximum cell concentration (4.2 g L^{-1}) was achieved at 36th hour, which corresponded to the maximum bioethanol concentration of 3.14 g L^{-1}. The spent sago bioethanol broth was harvested after 48 hours. In general, the broth is a heterogenous mixture of suspended solids, which was mostly made up of spent *S. cerevisiae and* brownish liquid fraction. Following the harvesting, the spent broth was centrifuged whereby the pellet was discarded whilst the liquid fraction, which is referred to as SBLW thenceforth, was characterised.

Table 1 presents the composition of SBLW in detail. The pH of SBLW was found to be 5.5. The acidic pH can be associated with the presence of organic acid such as lactic acid as quantified in this study. The residual bioethanol was found to be relatively low, which was only 0.083 g L^{-1}. The low amount of bioethanol in the broth was expected since most was removed during heating at its boiling point (80 ^{o}C). The main component of SBLW was found to be glycerol (3.25 g L^{-1}) followed by glucose (0.41 g L^{-1}).

To date, there is still little information available concerning the composition of spent broth generated from the production of bioethanol from sago fibre. Previous works reported the composition of liquid waste from industrial bioethanol produced from various agricultural sources such as sugarcane[15] and sugar beet molasses[19].

Table 1
Composition of SBLW

Parameter	Mean value (g L^{-1})
pH	5.50 ± 0.00
Glucose	0.41 ± 0.01
Glycerol	3.25 ± 0.23
Lactic acid	0.18 ± 0.05
Ethanol	0.08 ± 0.02

There are some similarities in terms of the characteristics of the liquid waste that can be observed between current work and those reports such as acidic pH (4-6) and the existence of glycerol and lactic acid. Glycerol is known as a by-product of ethanol biosynthesis when glucose is used as a carbon source[13]. The presence of glycerol in the liquid waste of bioethanol fermentation has also been reported in the literature[9,19]. The variation of glycerol concentration can be directly proportional to the initial concentration of glucose used in bioethanol fermentation.

Glycerol is a potential carbon source for certain microorganisms, thus its presence in bioethanol waste stream is seen as an advantage. The use of glycerol in bioethanol liquid waste as a medium for *Escherichia coli* fermentation expressing omega transaminase has been addressed by Suhaili et al[19].

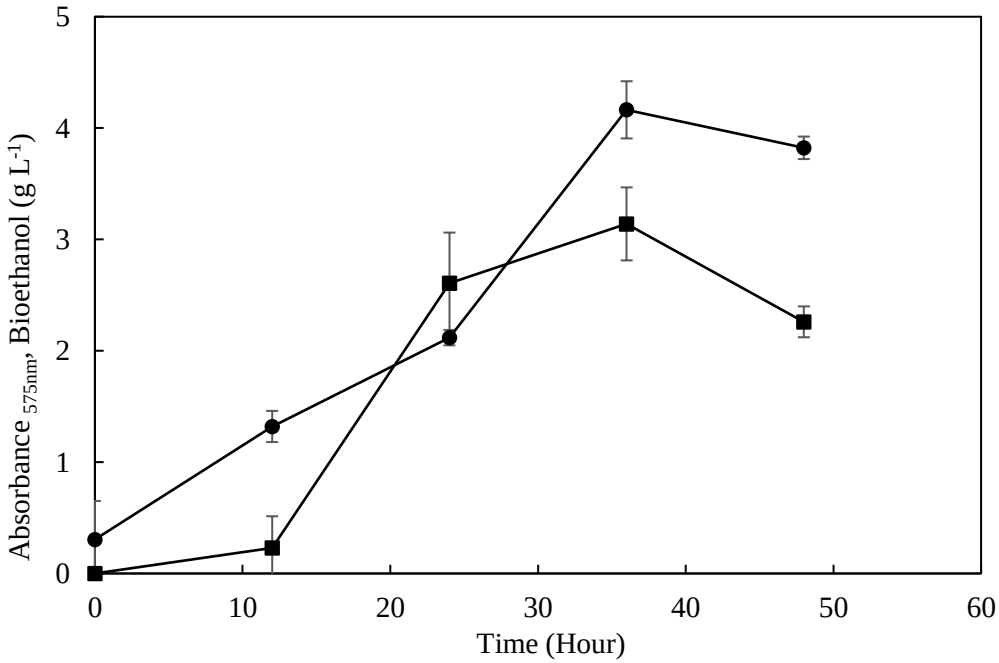

Fig. 1: Batch fermentation kinetics of *S. cerevisiae* cultured in shake flasks using SFH as a medium and the profile of the corresponding bioethanol produced. (●) Biomass; (■) bioethanol. Error bars indicate one standard deviation about the mean (n=3)

The glucose in SBLW represented the residual glucose from the bioethanol fermentation by *S. cerevisiae*. Previous reports rarely reported the presence of glucose in the bioethanol liquid waste stream as glucose normally serves as the main carbon source for bioethanol fermentation. Hence, its depletion upon the completion of the fermentation is very much expected. Different sources of the feedstock used for bioethanol fermentation such as sugar cane, beet molasses and sago fibre may influence the composition of the spent broth generated. This is due to the fact that each type of feedstocks has its own composition of carbon, nitrogen and trace elements that may affect the metabolism of the bioethanol producing yeasts.

Furthermore, variations in the procedures of bioethanol fermentation and distillation may also affect the characteristics of the spent broth generated[19]. Thus, these may explain the variations of the composition of the liquid waste generated after the bioethanol production. Apart from the components outlined in table 1, there were also other unknown compounds, reflected by the unknown peaks in the HPLC chromatogram, which require further research in future. In general, the findings on the composition of SBLW as highlighted in this work serve as an important stepping stone in understanding its potential as a feedstock for fermentation.

Feasibility of SBLW as a feedstock for recombinant *P. pastoris* fermentation expressing laccase: Fig. 2 depicts *P. pastoris* GS115 growth kinetics using SBLW prepared at different concentrations (40% to 100% (v/v)) and BMMH as a control. In general, the growth of *P. pastoris* GS115 was feasible in all fermentations using SBLW. Similar to the control experiment, fermentations using 40% and 50% (v/v) SBLW exhibited a logarithmic phase from day 0 to day 4, a stationary phase from day 4 to day 6 and a decline phase from day 6 to day 8.

On the other hand, in fermentations using 60% and 100% (v/v) SBLW, a sudden decrease in growth was observed after day 4. Although the actual reason is still unknown, this may be associated with the inhibitory effects of certain compounds that might be present in higher concentrations in 60% and 100% (v/v) SBLW compared to that in 40% and 50% (v/v) SBLW.

The maximum biomass concentration achieved from SBLW-based cultures was 13.9 g_{dcw} L^{-1} , which was attained from fermentations using 40% (v/v) SBLW. This represented 94% of the maximum biomass concentration achieved using BMMH as a medium (14.7 g_{dcw} L^{-1}). The good growth of *P. pastoris* GS115 in SBLW-based cultures suggests the potential of SBLW as a growth medium for the yeast. The feasibility of *P. pastoris* GS115 growth in SBLW-based fermentations was highly due to the presence of glycerol that serves as a carbon source for the yeast.

A number of works have addressed the use of bioethanol stillage for growing microorganisms such as *Spirulina maxima*[3], *Debaryomyces hansenii*[17], *Haloarcula marismortui*[15] and *E. coli*[19]. Nonetheless, to date, there has been no report on the use of SBLW for growing *P. pastoris*. Table 2 outlines the specific growth rate and maximum biomass concentration obtained from fermentations using BMMH and SBLW prepared at different concentrations.

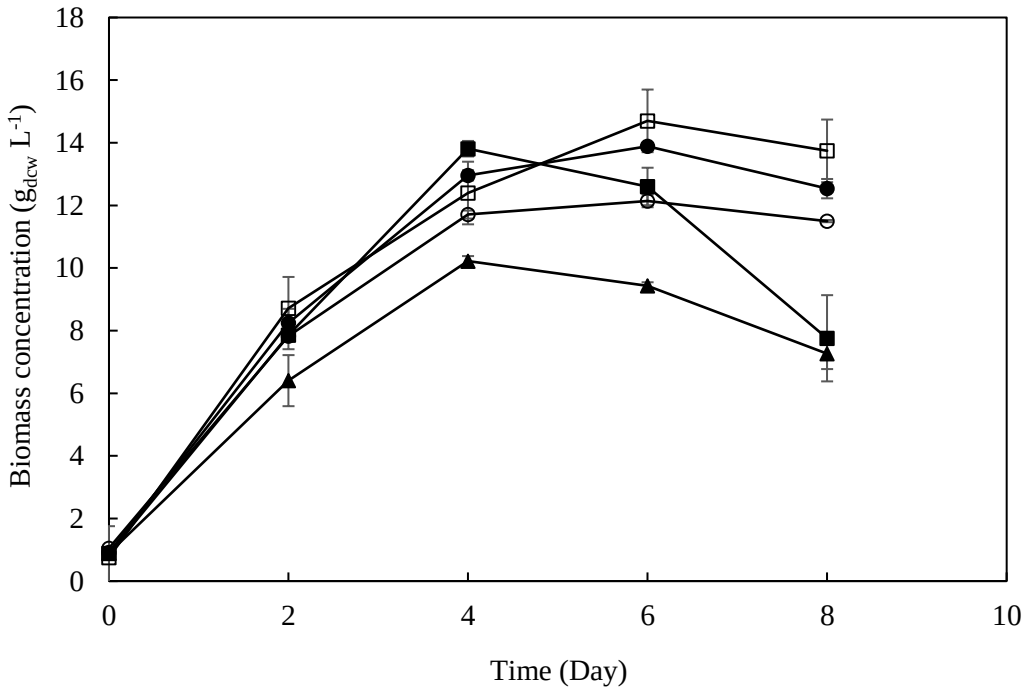

Fig. 2: Comparison of batch fermentation kinetics of *P. pastoris* GS115 when cultured using: (□) BMMH; (■) 100% (v/v) SBLW; (▲) 60% (v/v) SBLW; (○) 50% (v/v) SBLW; (●) 40% (v/v) SBLW. Error bars indicate one standard deviation about the mean (n=3)

Table 2
Kinetic parameters for *P. pastoris* GS115 growth when cultured on BMMH and SBLW at different concentrations

Medium	Specific growth rate, μ (h^{-1})	Maximum biomass concentration, X_{max} (g_{dcw} L^{-1})
BMMH (control)	0.10	14.70
100% (v/v) SBLW	0.13	13.81
60% (v/v) SBLW	0.10	10.22
50% (v/v) SBLW	0.11	12.14
40% (v/v) SBLW	0.10	13.89

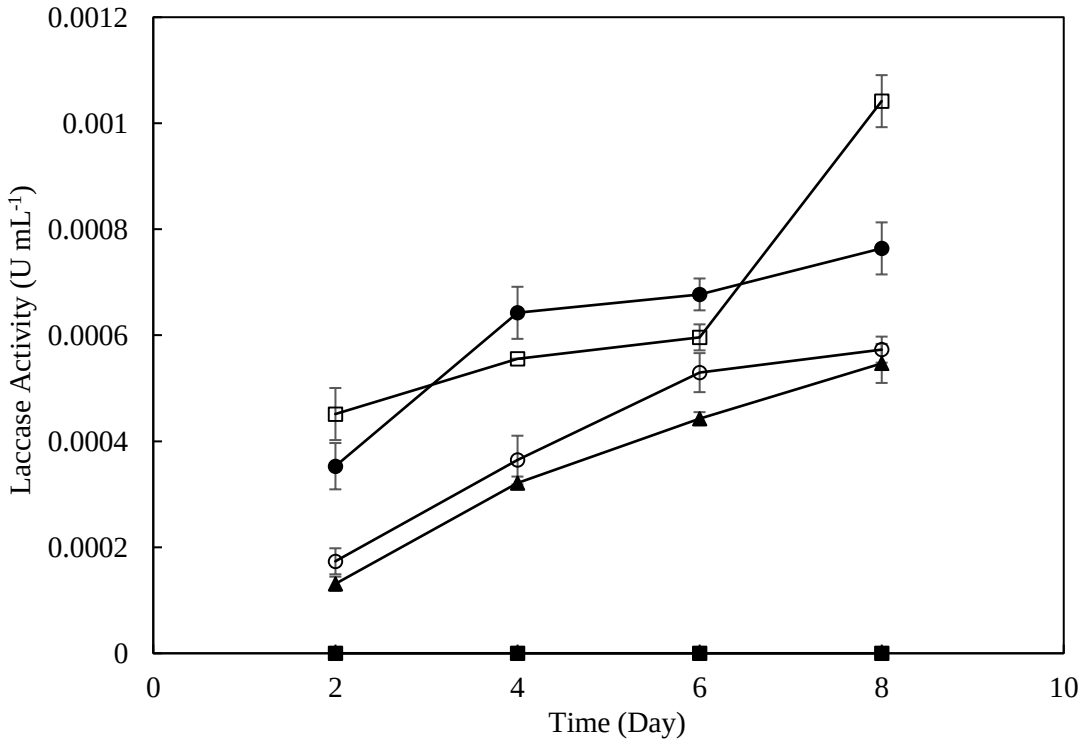

Fig. 3: Comparison of laccase activity from *P. pastoris* GS115 fermentations using: (□) BMMH; (■) 100% (v/v) SBLW; (▲) 60% (v/v) SBLW; (○) 50% (v/v) SBLW; (●) 40% (v/v) SBLW. Error bars indicate one standard deviation about the mean (n=3)

The corresponding laccase titres from fermentations illustrated in fig. 2 were also determined. Fig. 3 depicts the comparison of laccase activity obtained from fermentations using SBLW at different concentrations and BMMH as a control. The results showed that as the concentration of SBLW was increased from 40% to 100% (v/v), the overall activity of laccase was found to decrease. There was no activity of laccase detected from fermentations using 100% (v/v) SBLW. This might be due to various reasons such as high level of toxicity in concentrated SBLW and presence of by-proteins that may affect the laccase stability.

Further research is needed in order to better understand the association between the concentration of SBLW used and the corresponding laccase titre obtained.

The highest profile of laccase activity from fermentations using SBLW was exhibited by the culture that employed 40% (v/v) SBLW. The maximum laccase activity obtained in fermentations using 40% (v/v) SBLW was 0.00076 U mL^{-1} where this represented 73% of that attained using BMMH medium. The maximum peak of laccase activity attained using 40% (v/v) SBLW was also significantly higher than that obtained using 50% and 60% (v/v) SBLW. Our results here clearly showed that 40% (v/v) SBLW has a promising application as a feedstock for producing laccases by *P. pastoris* GS115.

Enhancement of laccase production by supplementing SBLW with yeast extract: Having established the optimal concentration of diluted SBLW for laccase production by *P. pastoris* GS115, subsequent work aimed to further enhance the production of laccases by supplementing the diluted SBLW with yeast extract (YE), a widely known nitrogen source that can enhance the fermentation yield. The best formulation of diluted SBLW (40% (v/v)) as concluded in the previous section was chosen for this work.

Different concentrations of YE in the range of 0.5% to 2.5% (w/v) were added to 40% (v/v) SBLW and the effect on *P. pastoris* GS115 growth and laccase production were investigated. Fig. 4 depicts the cell growth profile of *P. pastoris* GS115 when grown using BMMH, non-supplemented 40% (v/v) SBLW and 40% (v/v) SBLW supplemented with different concentrations of YE.

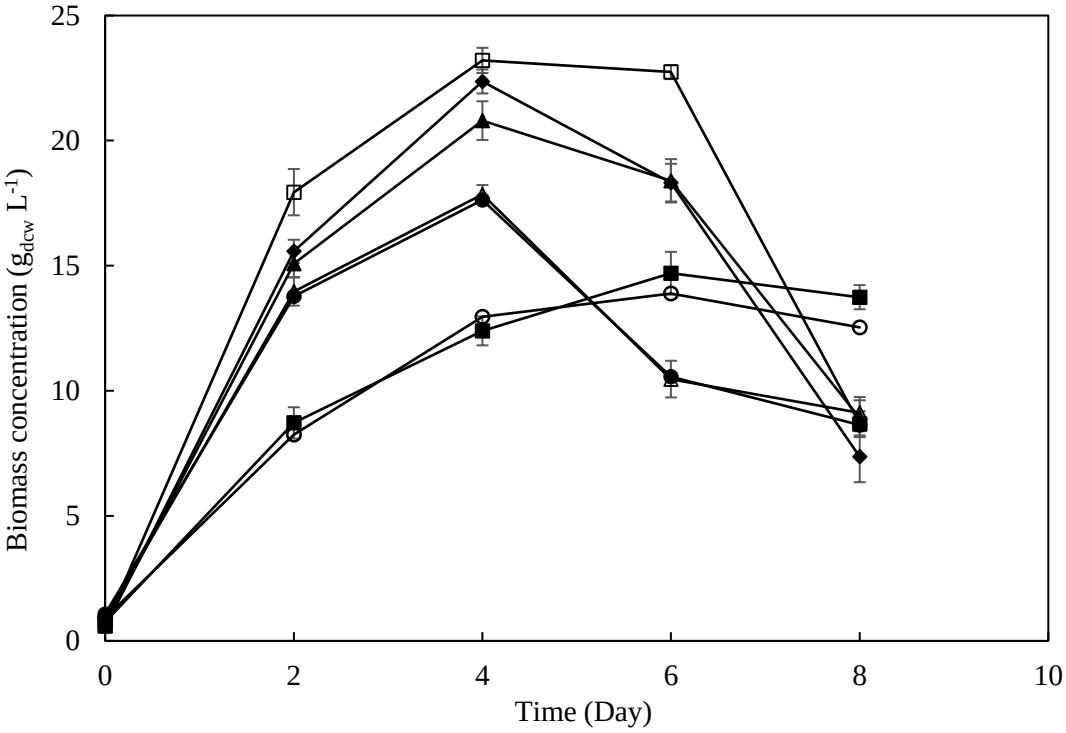

Fig. 4: Comparison of batch fermentation kinetics of *P. pastoris* GS115 when cultured using: (■) BMMH; (○) 40% (v/v) SBLW; (●) 40% (v/v) SBLW + 0.5% (w/v) YE; (Δ) 40% (v/v) SBLW + 1.0% (w/v) YE; (▲) 40% (v/v) SBLW + 1.5% (w/v) YE; (□) 40% (v/v) SBLW + 2.0% (w/v) YE; (♦) 40% (v/v) SBLW + 2.5% (w/v) YE. Error bars indicate one standard deviation about the mean (n=3)

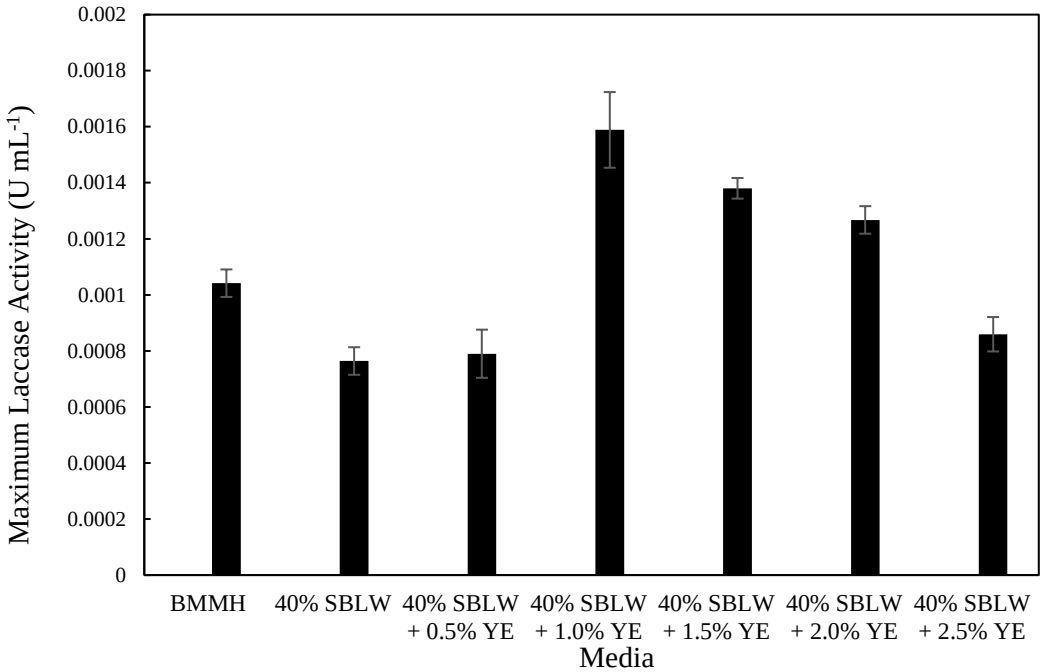

Fig. 5: Comparison of maximum laccase activity from *P. pastoris* GS115 fermentations using BMMH, diluted SBLW with no supplementation and diluted SBLW supplemented with yeast extract in the range of 0.5% to 2.5% (w/v). Error bars indicate one standard deviation about the mean (n=3)

It is shown that the specific growth rate increased dramatically to 1.9-2.5-fold when the 40% (v/v) SBLW was supplemented with 0.5 to 2.5 % (w/v) YE. In terms of maximum biomass concentration, the addition of YE within the tested range in this work, to 40% (v/v) SBLW has enhanced the peak by 1.3 to 1.7-fold in comparison to the non-supplemented culture. YE is known to contain growth factors and vitamins[21]. Hence the enhancement of cell growth and maximum biomass concentration in cultures that are supplemented with YE can be associated with the action of the aforementioned components.

Fig. 5 depicts the profile of maximum laccase activity from *P. pastoris* GS115 fermentations using 40% (v/v) SBLW supplemented with different concentrations of YE, non-supplemented SBLW and also BMMH as a control. The results demonstrated that in general, there was a notable benefit of supplementing the 40% (v/v) SBLW with YE. With regards to enzyme titre, the culture supplemented with 1.0% (w/v) YE was found to yield the highest titre of laccase (0.00159 U mL^{-1}), which represented 1.5 and 2.1-fold enhancement over BMMH and non-supplemented 40% (v/v) SBLW. The results obtained in this study were also higher than that reported by Larsson et al[12], Necochea et al[14] and Rivera-Hoyos et al[16]. Deprivation of nitrogen sources in cultivation media may result in a surge in protease activity[20]. It is believed that supplementation of SBLW with amino acid-rich compounds such as yeast extract can positively influence the protein production[4,10]. This is due to the inhibition of protease activity by the supplements that act as competing substrates, hence limiting the protease induction[22].

Conclusion

In conclusion, this work has demonstrated the feasibility of SBLW as a feedstock for recombinant laccase production by *P. pastoris* GS115. The use of 40% (v/v) SBLW was found to give the highest activity of laccase, which represented 73% of that achieved using standard synthetic medium (BMMH). Supplementation of 40% (v/v) SBLW with 1.0% (w/v) YE resulted in an enhancement of maximum biomass concentration and laccase titre by 1.2-fold and 1.5-fold respectively in comparison to that achieved using BMMH.

Future work should consider further optimisation of laccase production using 40% (v/v) SBLW supplemented with 1.0% (w/v) YE by considering more parameters such as other medium components.

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