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ORIGINAL ARTICLE

Improvement of biovanillin production with two-stage pH control strategy from lemongrass leaves hydrolysates using *Phanerochaete chrysosporium* ATCC 24725 in batch culture

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Abstract

The biovanillin production was influenced by varying the culture pH via single control strategy conducted by separate experiments during the fermentation processes. Highest biovanillin production (124 mg/L) with 32% molar yield at culture pH 6.0 from the one-stage control method was observed. Specific growth rates (μ) of the *Phanerochaete chrysosporium* and biovanillin production decreased by decreasing the culture pH from 6.0 to 3.5, which indicated that lower culture pH was not adequately apposite for biovanillin production using *Phanerochaete chrysosporium* in a 2-L stirred tank bioreactor. The development of two-stage control strategies had improved the biovanillin production (131 mg/L) and cell concentration (13.0 g/L) by about 6 and 5%, respectively. Therefore, the most influential control strategy for higher biovanillin production was discovered not to control the culture pH of the fermentation during active growth phase of the *Phanerochaete chrysosporium*, while the production phase should be controlled at pH 6.0.

Keywords Batch culture · Biovanillin · Lemongrass leaves hydrolysates · *Phanerochaete chrysosporium* ATCC 24725 · pH control strategy · 2-liter stirred tank bioreactor

1 Introduction

Biovanillin is regarded as vanillin obtained through the application of biotechnological approaches to extract precursors, like ferulic acid, isoeugenol, guaiacol, and eugenol, from various agro-based residues including rice bran and stem, maize stem, lemongrass leaves, palm oil bunch, and sugarcane bagasse, using microorganisms like *Phanerochaete chrysosporium* ATCC 24725 [1, 2]. The *Phanerochaete chrysosporium* ATCC 24725 was reported with capability of liberating enzymes like laccases, lignin peroxidase, and manganese peroxidase with high affinity to degrade lignin (which serves as a barrier) from the biomass, utilizing nitrogen and carbon that were used as substrates for the fungus growth and metabolism [3, 4]. The *P. chrysosporium* ATCC 24725 was equally reported to release vanillin synthase (*VpVAN*), which is an enzyme that facilitates the direct transformation of ferulic acid into biovanillin [4, 5]. Due to the lower yield of the biovanillin because of high toxic effects of the other precursors during fermentation process, as well as chemical similarity of ferulic acid (FA) to vanillin, the use of FA obtained from agricultural residues was established among the best routes

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for biovanillin production [6–8]. The biovanillin has been used as flavor additives in the production of several products in food industries, cosmetics, agriculture, drugs, and household [9]. The laboratory and industrial production of biovanillin could be improved by selecting an ideal research design with appropriate composition of the culture medium, together with the optimum physiological factors [1, 10]. This could be due to their substantial effects on the *P. chrysosporium* and eventual release of the vanillin synthase for the biovanillin production [11].

The biovanillin production was determined to be influenced significantly by the combination of parameters like pH, temperature, and incubation time during optimization processes with central composite design [12]. Thus, pH of culture medium appears as one of the vital factors of the medium components for fermentation process, since change in pH value is among the major problems faced in biovanillin production using a bioreactor [13]. The effect of pH on various bioproduct production was reported of being a vital parameter [14]. Previous researchers have revealed that negative pH effect on the culture medium had tremendously contributed to the inefficiency of the fermentation due to low cell concentration and prolonged culture lag phase coupled with microbial and product inhibitions [15, 16]. Efforts had since been put in place by various researchers to achieve utmost bioproduct production via employing numerous strategies like elimination or reduction of product toxicity and manipulation of the culture pH medium [14, 15, 17]. Therefore, appropriate selection and application of strategy for pH control during fermentation could lead to provision of favorable culture conditions, which would facilitate the microbial growth and enhance the release of enzymes pertinent to the production of respective bioproducts including biovanillin [18, 19]. Several researchers have investigated the influence of nutrient composition together with other physical parameters for bioproduct formation. In most of the reported studies, the influence of pH for bioproduct formation was achieved by monitoring the culture pH at single stage during the whole fermentation processes. Furthermore, some researchers have been documented on the influence of monitoring the culture pH at various stages during both growth phase and production phase to produce kojic acid, citric acid, and antibiotics. However, there was no such research on biovanillin production. Therefore, this research had explored the effect of pH control by developing various control strategies at both growth and production phases toward biovanillin production from lemongrass leaves hydrolysates using a 2-L stirred tank bioreactor.

2 Materials and methods

2.1 Microorganism and inoculum preparation

Phanerochaete chrysosporium ATCC 24725 was used for the biovanillin transformation from ferulic acid obtained in lemongrass leaves hydrolysates. The preparation of the inoculum was performed in accordance with the method reported by Hussin et al. [20], by growing the fungal strain onto Potato Dextrose Agar at 30 °C for 7 days. Harvest of the spore was conducted using 1% v/v from tween-80 by centrifugation at 4000 rpm for 20 min [12, 20]. The harvested spore was diluted with purified distilled water to get the required inoculum sizes prior to the inoculation.

2.2 Medium preparations and batch culture setup in the bioreactor

A total of 39 g of the PDA powder (as growth medium) was suspended into 1 L of distilled water, followed by 1-min warming and agitation thoroughly to enable the contents to dissolve completely before finally used for the fungus growth. The medium used for the biovanillin production was as described by Tilay et al. [1] with modifications [12, 20]. The components of the medium include in g/L of KH_2PO_4 (0.2), $MgSO_4.7H_2O$ (0.5), $CaCl_2.2H_2O$ (0.132), thiamine hydrochloride (0.0025), glucose (20), ammonium chloride (4.0), and yeast extract (0.5). The medium components (except glucose which was filter-sterilized) were dissolved completely into the optimized lemongrass leaves hydrolysates and autoclaved at 121 °C for 30 min., after appropriate adjusting the pH to the required value. The chemicals used were purchased from Sigma-Aldrich (M) Sdn Bhd, Bandar Sunway, Subang Jaya, 46150, Petaling Jaya, Selangor, Malaysia.

All the experiments were performed in a 2-L stirred tank bioreactor (Biostat® B, Germany, Sartorius BBI system) with a running volume of 1500 mL. The bioreactor comprises of a cylinder-shaped vessel made from double-rounded glass with a dynamic shaft at the center that holds 6-blade impellers which permits proper mixing of the culture medium even at lower level of agitation speeds. After preparation of the production medium, it was then transferred into a double jacketed borosilicate glass of the bioreactor and covered loosely with head plate of stainless steel prior to the autoclave. The pH electrode probe (Mettler Toledo, Switzerland) calibration was performed using standard pH (7.0 and 4.0) buffers, prior to the appropriate adjustment of the medium pH to the required value. Compressed nitrogen gas was introduced gradually into the bioreactor to remove some of the oxygen present in the culture medium before and after the sterilization, to stabilize the culture environment before inoculation. Eventually, inoculation of the sterilized bioreactor housing 1500 mL of the production medium was performed with 15 mL (1% v/v) from the initial spore suspension of 1.0×10^7 spore/mL.

2.3 Set up for pH control strategy in 2-L stirred tank bioreactor

Initially, screening of significant parameters (temperature, time, pH, inoculum size, ammonium chloride, yeast extract,

agitation speed, and ferulic acid concentration) towards the biovanillin production was performed using 2-level factorial design (2-LFD) in shake flasks. Four parameters (temperature, time, pH, and ferulic acid concentration) were found to significantly influence the biovanillin production. Thus, optimization of the biovanillin production was conducted with these four significant parameters in shake flasks using CCD (data not shown). Laboratory experiments with the bioreactor for pH control strategy were succeeded, which was initiated without the control of the pH (initial pH 6.0). The data obtained were used in the development of the successive one-stage control strategy. During the one-stage control strategy, the culture pH was controlled at 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, and 3.5 differently. Subsequently, the data obtained from the one-stage control strategy were used in developing the second-stage control strategy. The two-stage control strategy was set up as follows: the pH was not controlled during growth phase (initial pH 6.0), then was switched to 6.0 during the production phase (stategy1); the pH was not controlled during growth phase (initial pH 6.0), then was switched to 6.5 during the production phase (strategy2); the pH was not controlled during growth phase (initial pH 6.0), then was switched to 5.5 during the production phase (strategy3); the pH was not controlled during growth phase (initial pH 6.0), then was switched to 5.0 during the production phase (strategy4); the pH was controlled at 6.0 during growth phase, then was not controlled during the production phase (strategy5); the pH was controlled at 5.5 during growth phase, then was not controlled during the production phase (strategy6); the pH was controlled at 6.0 during growth phase, then was controlled at 5.5 during the production phase (strategy7); the pH was controlled at 5.5 during growth phase, then was controlled at 6.0 during the production phase (strategy8). Figure 1 depicts the overall summary of both the one-stage and two-stage pH control strategies toward biovanillin production in 2-L stirred tank bioreactor.

The whole pH control process during the experiments was performed using 2 M HCL and 2 M NaOH that were previously sparged with nitrogen gas and autoclaved at 121 °C for 30 min in 500-mL schott bottles. The addition of the acid or alkali into the bioreactor as a measure for the pH control was achieved by using an in-built peristaltic pump (Econo Gradient Pump from Bio-Rad) linked with a butyl tubing from the bioreactor's control panel to the head plate of the stirred tank. The calibration and monitoring of temperature, dissolved oxygen, agitation speed, and pH were all performed by a hardware control system equipped onto the bioreactor with a touchpad set up interface.

2.4 Analysis procedure

Samples were withdrawn from the bioreactor at every 6 h for subsequent analysis. The phenolic compounds were analyzed with HPLC-DAD at 320, 280, and 254 nm wavelengths, Agilent eclipse XDB-C18 column (5 μm, 0.5 × 150 mm) and a flow rate at 20 μL/min [12, 20]. Quantification of the compounds was performed using external standards. 50:50 ratio of the two mobile phases (acetonitrile:acetic acid:water, i.e., 20:2:78, and water:acetic acid, i.e., 98:2) was used as described by Hussin et al. [20]. Glucose concentration via total reducing sugar was determined with dinitrosalicylic (DNS) reagent as described by Miller [21] with slight modification [22]. Standard curve was developed with glucose monohydrate for the quantification of the total reducing sugar from the sample. Cell concentration was determined using oven-dry mycelia weight with cellulose acetate membrane (0.2 μm), which was used to drain other liquid parts of the medium composition, leaving only wet cell biomass on the membrane.

3 Results and discussion

3.1 Biovanillin production in batch fermentation using one-stage pH control strategy

The profiles for biovanillin production and fermentation kinetics by *Phanerochaete chrysosporium* ATCC 2472 in the 2-L stirred tank bioreactor with varying culture pH are provided in Table 1. The biovanillin production was influenced by varying the culture pH of the experiments using the *P. chrysosporium* ATCC 2472 in 2-L stirred tank bioreactor as described in Table 1. The cell biomass concentration of the fungus decreased with decrease in the culture pH of the medium from 6.0 to 3.5, with a short lag phase noticed in all the experiments apart from culture pH at 4.5, 4.0, and 3.5, where the lag phases of the fungal growth were relatively prolonged (data not shown). Unfavorable pH set up of culture medium during fermentation was found among the key factors that caused a delay in the normal growth of microorganisms, which ultimately affected the entire fermentation processes [15, 23–25]. However, there were no significant differences in the pattern of the fungal growth at culture pH 6.5, 6.0, and without pH control (initial pH 6.0) and pH 5.5 and pH 5.0, and the fungal growth attained stationary phase during the fermentation period at about 42 h, 48 h, and 54 h, respectively (data not shown), during which the cell biomass has reached maximum concentration at the range of 12.43–5.91 g/L (Table 1).

Highest biovanillin production (124 mg/L) with 32% molar yield and 1.6 mg/L/h maximum productivity was recorded when the pH culture was controlled at 6.0 during the fermentation process. This indicates that the culture pH is adequate to support the microbial growth and subsequent release of pertinent enzymes for biovanillin transformation [1, 2, 26]. Accordingly, increasing the culture pH to 6.5 did not significantly influence the biovanillin production (122 mg/L)

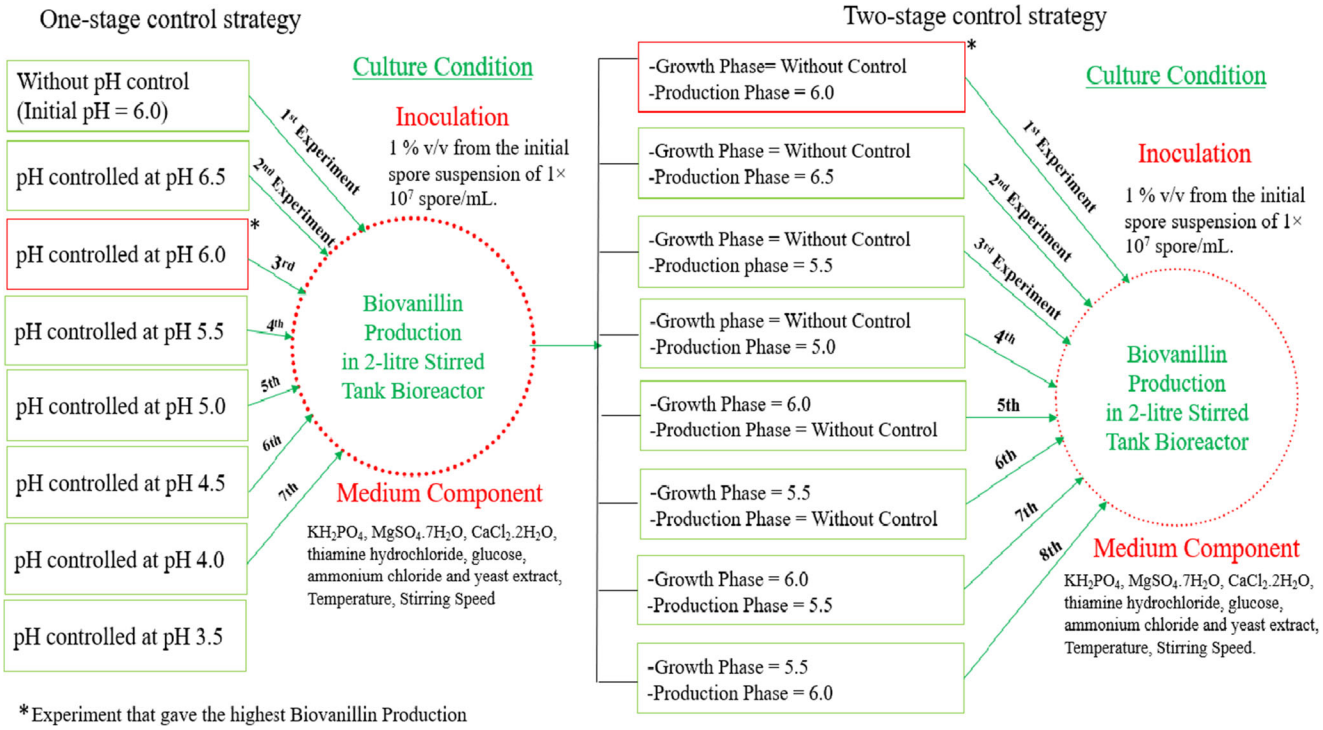

Fig. 1 Overall summary of the pH control strategies toward biovanillin production in 2-L stirred tank bioreactor

(Table 1). More so, there was no significant difference from the biovanillin production when the culture pH was controlled at 5.5 (94 mg/L with 24% molar yield) to that culture pH without control (with initial pH at 6.0) (98 mg/L with 25% molar yield). Therefore, the values of their maximum biomass formation (X_{max}, 11.09 g/L and 11.14 g/L), both yield coefficients of biomass ($Y_{x/s}$ 467 mg/g and 465 mg/g), and biovanillin production ($Y_{p/s}$ 3.9 mg/g and 4.1 mg/g) towards utilizing glucose are

Table 1 Fermentation kinetics of single-phase pH control strategy towards biovanillin production

Kinetic factors	Without pH control	pH at 6.5	pH at 6.0	pH at 5.5	pH at 5.0	pH at 4.5	pH at 4.0	pH at 3.5
Maximum production, P_{Max} (mg/L)	98	122	124	94	72	56	37	18
Initial ferulic acid conc. (mg/L)	504	500	502	504	500	504	500	500
Final ferulic acid conc. (mg/L)	47	22	21	66	111	160	254	267
Ferulic acid converted to biovanillin (%)	21	26	26	21	19	18	15	11
Molar yield (%)	25	31	32	24	18	16	9.0	5.0
Initial glucose conc. (g/L)	24.99	24.86	24.86	24.95	24.99	24.99	24.88	24.94
Final glucose conc. (g/L)	0.55	0.11	0.18	0.68	0.37	1.62	4.84	7.61
Total rate of glucose consumed (%)	97.80	99.56	99.28	97.27	98.52	93.52	80.55	69.49
X_{max} (g/L)	11.14	12.43	12.14	11.09	11.36	10.22	6.84	5.91
Specific growth rate, μ (h^{-1})	5.3×10^{-2}	5.5×10^{-2}	5.3×10^{-2}	4.9×10^{-2}	4.6×10^{-2}	3.4×10^{-2}	2.8×10^{-2}	2.2×10^{-2}
Max. specific growth rate, μmax (h^{-1})	0.106	0.110	0.106	9.8×10^{-2}	9.2×10^{-2}	6.8×10^{-2}	5.6×10^{-2}	4.4×10^{-2}
$Y_{x/s}$ (mg/g)	467	506	499	652	469	446	352	345
$Y_{p/s}$ (mg/g)	4.10	4.90	5.10	3.90	3.10	2.50	2.20	1.30
$Y_{p/x}$ (mg/g)	9.10	10.3	10.8	8.90	6.70	5.80	5.60	3.30
Maximum productivity (mg/L/h)	1.20	1.50	1.60	1.10	1.00	0.80	0.60	0.30
Overall productivity (mg/L/h)	0.67	1.21	1.22	0.88	0.57	0.40	0.30	0.10
Doubling time, td (h^{-1})	13	13	13	14	15	21	25	32
t (h)*	84	84	78	84	72	66	66	60

*Time at which the biovanillin production reached maximum. *Y* yield coefficient

barely the same (Table 1). This could perhaps be attributed to the decrease of the pH value to 5.6 in the experiment without pH control after 66 h of the fermentation, which was almost the same pH value to that of the experiment during which the pH was controlled at 5.5. Previously conducted research has equally recorded successes in the biovanillin production by utilizing various substrates [27, 28]. For example, Karode et al. [29] have used the strain of *Phanerochaete chrysosporium* NCIM 1197 for the biovanillin production after utilizing various agro-based residues as substrates. From the results of their experiment, maximum biovanillin concentration reached 170 mg/L at 96 h with glucose and ferulic acid. A slight decrease in the biovanillin concentration (166 mg/L) was observed when only ferulic acid was added to the components of the culture medium, but time taken (96 h) to reach maximum biovanillin production was the same. The biovanillin production (114 mg/L) was also observed to decrease by replacing the glucose with starch. Also, the use of potato dextrose broth and groundnut shell during the biovanillin biotransformation had reduced the biovanillin concentration (55 mg/L) by 3.1-folds. Moreover, combining glucose with groundnut shell without ferulic acid and using only groundnut shell being added to the culture medium were both observed to decrease the biovanillin concentration by 4.6- and 5.7-folds, respectively. These have shown that ferulic acid could serve as a better precursor for biovanillin transformation, since combining the ferulic acid with simple sugar like glucose has resulted to the upsurge in the biovanillin concentration during the fermentation processes.

The biovanillin production was decreased by decreasing the culture pH from 6.0 to 3.5, which also indicated that lower pH values were not adequately suitable for biovanillin production using *Phanerochaete chrysosporium* ATCC 24725 with lemongrass leaves hydrolysates [1, 20]. The maximum specific growth rate (μ_{max}, 0.106 h^{-1}) of the fungus when the culture pH was not controlled (with initial pH of 6.0) was equivalent to that experiment when the culture pH was controlled at 6.0. This could presumably be due to maintaining the culture pH value at 6.1 (which is nearly the same with the experiment under controlled pH of 6.0) during the fungal active growth at the experiment when the culture pH was not controlled (initial pH of 6.0). The rate of microbial growth and that of the fermentation processes were discovered to be vested on the ability of microorganisms to adjust and adopt to their culture conditions [18, 30, 31].

However, the maximum specific growth rate (μ_{max}) of the fungus was observed to decrease with the decreasing culture pH from 6.5 to 3.5, as indicated in Table 1. Highest cell concentration (X_{max}, 12.43 g/L) was noticed when culture pH was controlled at 6.5. This could be affirmed by the high yield coefficient of the biomass formation ($Y_{x/s}$, 506 mg/g) with low yield coefficient of the biovanillin production ($Y_{p/s}$, 4.90 mg/g) as compared with that when the culture pH was controlled at 6.0, where the yield coefficient of the biomass formation ($Y_{x/s}$, 499 mg/g) was lower and the yield coefficient of the biovanillin production ($Y_{p/s}$, 5.10 mg/g) was higher. In another word, the fungi consumed glucose (99.56%) more towards microbial cell growth and metabolism when the culture pH was controlled at 6.5 than for biovanillin production. But when the pH was controlled at 6.0, the fungus consumed glucose (99.28%) more for biovanillin production ($Y_{p/s}$) than for cell growth and metabolism ($Y_{x/s}$) (Table 1). Subsequently, this is supported by the higher values of yield coefficient of biovanillin related to biomass formation ($Y_{p/x}$, 10.8 mg/g), maximum and overall productivity values of 1.60 mg/L and 1.22 mg/L/h, respectively, as equated to other experiments when the culture pH was controlled at 6.5, 5.5, 5.0, 4.5, 4.0, 3.5, and without pH control (at initial pH 6.0) (Table 1).

The similarities in the doubling time of the fungus (13 h) at culture pH of 6.5, 6.0, and without pH control were confirmed by the fungal specific growth rate (μ) 0.055 h^{-1}, 0.053 h^{-1}, and 0.053 h^{-1}, respectively, which indicated that the fungal growth was not significantly influenced by varying the pH at these values at the onset of the experiments, particularly during the fungal active growth periods. In another way, the fungi had almost similar time (13 h) to double their number at the said culture pH during the fungal active growth periods. Yet, the slowest doubling time (32 h) was observed when the culture pH was controlled at 3.5, which was affirmed by the lower specific growth rate, μ (0.022 h^{-1}) indicating that the fungi were not adequately active at lower pH values [1, 29]. Therefore, since the biovanillin transformation coupled with the fungal cell concentration was greatly favored at high pH value, and the fact that the whole fermentation process was observed to undergo two different stages, growth phase and production phase, there is a need to further develop a two-phase control strategy of the pH to explore the potentiality of the *Phanerochaete chrysosporium* ATCC 24725 with lemongrass leaves hydrolysates in 2-L bioreactor towards the biovanillin production.

3.2 Biovanillin production in batch fermentation using two-phase pH control strategy

Fermentation kinetics for the two-phase pH control strategy in 2-L stirred tank bioreactor towards biovanillin production was initially performed following the results of the one-phase pH control strategy. The fermentation performance together with the kinetics evaluation for the biovanillin production with the culture pH controlled and without control at two successive phases of the fermentation process are provided in Table 2. Highest biovanillin production (131 mg/L) was recorded at strategy1 with a molar yield of 33% and maximum productivity of 1.80 mg/L. An increase in the culture pH at strategy2 by switching the culture pH to 6.5 during the production phase did not significantly influence the biovanillin production, since 130 mg/L of the biovanillin was obtained with a molar

yield of 33% and maximum productivity of 1.70 mg/L. However, highest cell concentration (13.0 g/L) was observed at strategy2 as compared with strategy1 where the cell concentration reached maximum of 12.52 g/L. Further comparison between the two strategies (1 and 2) revealed that strategy1 favored product formation slightly, whereas strategy2 had considered the formation of cell to some extent. This could be ascertained as in the case of one-stage control strategy, in which higher yield coefficient for the biomass formation ($Y_{x/s}$, 519 mg/g) with lower yield of coefficient for the biovanillin formation ($Y_{p/s}$, 5.20 mg/g) was discovered at strategy2, while the yield coefficient of the biomass formation ($Y_{x/s}$, 503 mg/g) was lower and the yield coefficient for the biovanillin formation ($Y_{p/s}$, 5.30 mg/g) was slightly higher with 1.02-folds. Therefore, their relationships could further be explained based on glucose consumption with regard to the formations of both fungal cell and biovanillin under these monitored culture conditions. Much glucose utilization (100%) for growth and metabolism was observed ($Y_{x/s}$, 519 mg/g) at strategy2 as compared with 99.96% of the glucose being consumed for growth and metabolism ($Y_{x/s}$, 503 mg/g) as indicated in Table 2. It could equally be interpreted further as 519 mg of the fungal cell was formed per unit gram of the glucose being utilized in stategy1, while 503 mg of the cell was harvested per every gram of the consumed glucose. Fast utilization of substrate for microbial growth and cellular metabolism was described to take place in any fermentation process due to the proper combination of the culture conditions [23, 32–34]. However, adequate care has to be taken in the selection of the best combination that could facilitate the formation of the required products more than the cellular building [25, 31, 35].

Based on the experimental results as presented in Table 2, there are no significant differences on the maximum cell concentrations observed in strategy1 (12.52 g/L) and strategy5 (12.36 g/L), as well as that observed in strategy3 (11.56 g/L) and strategy4 (11.68 g/L). Therefore, this could further be described and testified by the investigation of other components of the evaluated kinetic parameters as provided in Table 2. The similarity and closeness in the values of the

Table 2 Fermentation performance and kinetic evaluation of biovanillin production using two-phase control strategy

Kinetic factors	STG1	STG2	STG3	STG4	STG5	STG6	STG7	STG8
Maximum production, P_{Max} (mg/L)	131	130	117	66	83	54	80	104
Initial ferulic acid concentration (mg/L)	501	503	501	501	502	503	501	502
Final ferulic acid concentration (mg/L)	13	16	25	113	49	137	85	34
Ferulic acid converted to biovanillin (%)	27	27	25	17	18	15	19	22
Molar yield (%)	33	33	29	17	21	14	20	26
Initial glucose concentration (g/L)	24.86	24.99	24.84	24.84	24.99	24.86	24.99	24.86
Final glucose concentration (mg/L)	1.00	0.00	3.00	2.00	1.00	1.00	1.00	0.21
Glucose consumed (%)	99.96	100.0	99.88	99.92	99.96	95.98	99.96	99.16
Maximum Cell Concentration, X_{max} (g/L)	12.52	13.00	11.57	11.68	12.36	10.68	12.10	11.23
Specific growth rate, μ (h^{-1})	5.5×10^{-2}	5.6×10^{-2}	5.5×10^{-2}	5.6×10^{-2}	5.5×10^{-2}	5.0×10^{-2}	5.4×10^{-2}	4.9×10^{-2}
Max specific growth rate, μmax (h^{-1})	0.110	0.112	0.110	0.112	0.110	0.100	0.108	9.8×10^{-2}
Yield coefficient of biomass to glucose, $Y_{x/s}$ (mg/g)	503	519.	476	478	500	459	491	465
Yield coefficient of biovanillin to glucose, $Y_{p/s}$ (mg/g)	5.30	5.20	4.50	2.80	3.30	2.30	3.20	4.30
Yield coefficient of biovanillin to biomass, $Y_{p/x}$ (mg/g)	11.0	11.4	10.4	5.70	7.20	5.20	6.90	9.30
Maximum productivity (mg/L)	1.80	1.70	1.50	1.00	1.00	1.20	1.00	1.03
Overall productivity (mg/L/h)	1.08	1.09	1.01	0.49	0.72	0.40	0.67	0.90
Doubling time, td (h^{-1})	13	12	13	12	13	14	13	14
t (h)*	72	78	78	66	78	72	78	78

*Time at which the biovanillin production reached maximum. *STG* strategy

STG1 = pH was not controlled during the growth phase, then switched to pH 6.0 during production phase

STG2 = pH was not controlled during the growth phase, then switched to pH 6.5 during production phase

STG3 = pH was not controlled during the growth phase, then switched to pH 5.5 during production phase

STG4 = pH was not controlled during the growth phase, then switched to pH 5.0 during production phase

STG5 = pH was controlled at 6.0 during the growth phase, then it was not controlled at production stage

STG6 = pH was controlled at 5.5 during the growth phase, then it was not controlled at production stage

STG7 = pH was controlled at 6.0 during the growth phase, then switched to pH 5.5 during production phase

STG8 = pH was controlled at 5.5 during the growth phase, then switched to pH 6.0 during production phase

specific growth rate of 0.055 h^{-1} observed in strategy1, strategy5, and strategy3 and 0.056 h^{-1} in strategy4, respectively, could justify the close relationships that existed between the mentioned experimental strategies. More so, the close relationships between the highlighted strategies could further be viewed by the slighter variation that was observed in the values of the yield coefficient of biomass formation ($Y_{x/s}$, 503 mg/g) obtained in strategy1 and 500 mg/g obtained in strategy5 with a variation of only 0.0037 (0.74%), and with 476 mg/g obtained in strategy3 and 478 mg/g obtained in strategy4 with a disparity of only 0.0026 (0.54%). Thus, it could be inferred based on the identified relationships between the strategies that nearly the same amount of glucose was consumed by the fungus for growth and cellular metabolism to enable them to attain average maximum specific growth rate of 0.110 h^{-1}. Also, it could further interpret the observed relations of these experiments that the established differences between these various strategies did not influence the rates of glucose consumption as well as the growth of the fungus. Rosfarizan et al. [36] have reported that variation of culture pH from various control strategies did not significantly influence the rate of glucose consumption during growth phases of the fungus, and the growth patterns from the various pH control strategies were barely the same.

These similarities and closeness of the identified determinants from the fungal growth phase as presented in Table 2 could equally be investigated by considering the time taken by the fungus to double their number during the fermentation processes. Thus, the similarities in the observed time of 12 h (in strategy2 and 4), 13 h (in strategy1, 3, 5, and 7), and 14 h (in strategy6 and 8) have been affirmed by the closeness in maximum specific growth rates of 0.112 h^{-1}, 0.110 h^{-1}, and 0.100 h^{-1} of the fungus, respectively. The statement could be reframed further as it took 12 h, 14 h, and 13 h for the fungi to double their number during the fermentation process in strategy2 and 4; strategy6 and 8; and strategy1, 3, 5, and 7, and the fungi reached maximum specific growth rate of 0.112 h^{-1}, 0.110 h^{-1}, and 0.100 h^{-1}, respectively. Therefore, this has specified that the rate of glucose consumption and the growth of the fungi was not significantly affected by the fluctuation of the pH as indicated from the corresponding various developed strategies during the active growth phases, as was reported by Rosfarizan et al. [36]. However, the situation was not similar in the case of biovanillin production, particularly during the production phase of the fermentation processes. Such similarities and closeness between the evaluated strategies based on the kinetics performance were not so intense, as can be viewed in Table 2. For example, the biovanillin production has reached a maximum concentration of 0.131 g/L with yield coefficient of biovanillin formation ($Y_{p/s}$, 5.30 mg/g) in strategy1, whereas maximum concentration of the biovanillin with yield coefficient of biovanillin formation ($Y_{p/s}$, 3.30 mg/g) was obtained in strategy5. Therefore, by comparison between the two strategies (1 and 5) there are quite variations of up to 0.048 (57.83%) and 0.002 (60.61%) from the obtained results of the maximum biovanillin production and yield coefficient of the biovanillin formation in both strategy1 and strategy5, respectively.

The same trend of these variations was observed in strategy3 and strategy4, where 117 mg/L and 66 mg/L with 4.50 mg/g and 2.80 mg/g of the maximum biovanillin productions and yield coefficient of biovanillin formations were recognized with variations of 0.051 (77.27%) and 0.0017 (60.71%) in both strategy3 and strategy4, respectively. It could be deduced from these experimental analyses that, despite similarities observed from the growth performance of the fungus during the growth phase, switching of the pH to different values during the production phase had enormously influenced the production of the biovanillin (Table 2). Rosfarizan et al. [36] had described that, despite insignificant rates of glucose consumption and growth observed during the growth phases of fungus, varying the pH according to the developed strategies during production phases was found to significantly influence the performance of the fermentation accordingly. The ability for the microorganisms to adopt to wide range of pH had gave them the opportunity to survive in various pH control strategies, which also enables them to demonstrate a very close relation in their growth pattern during the active growth phases, but switching to various pH during the production phases was discovered to influence the product formation significantly [14, 16, 36].

The highest biovanillin production of 131 mg/L with overall productivity of 1.08 mg/L/h was attained from the two-phase control in strategy1 with an improvement of 6% higher as related to the highest biovanillin produced (124 mg/L with overall productivity of 1.22 mg/L/h) using one-stage control strategy from pH controlled at 6.0. However, the time taken for the biovanillin concentration to reach maximum (72 h) was 6 h shorter than when using one-stage control strategy. As such, lower overall productivity was observed (Table 2). Based on the above comparison, the fermentation conditions provided in strategy1 from the two-phase control were more favorable to enable the secretion of adequate vanillin synthase that facilitated the ferulic acid bioconversion to biovanillin.

3.3 Comparison for the performance of pH kinetics of the biovanillin production

Comparison of the performance of pH control kinetics for the biovanillin production by *Phanerochaete chrysosporium* ATCC 24725 in shake flasks and bioreactor (using one-phase and two-phase pH control strategies) is presented in Table 3. The biovanillin production was enhanced from the fermentation using two-phase pH control strategy, where 131 mg/L of the biovanillin production with 33% molar yield and 1.80 mg/L maximum productivity were observed. Although,

the biovanillin production from fermentation using one-stage pH control was low (124 mg/L with 32% molar yield and 1.60 mg/L/h) when compared with that of two-stage pH control, improvement of the biovanillin production was recorded by 1.27- and 1.33-folds from fermentations without control of the pH (with initial pH 6.0) and that conducted in shake flasks, respectively (Table 3).

Performance of the kinetics was further evaluated by considering the concentration of the cell biomass and yield coefficients for biovanillin production and biomass formation. Higher concentration of the fungal cell biomass, X_{max} (12.52 g/L), with yield of biomass formation through glucose consumption, $Y_{x/s}$ (503 mg/g), yield of the biovanillin via glucose utilization, $Y_{p/s}$ (5.30 mg/g), and the contribution of the cell biomass towards the biovanillin production, $Y_{p/x}$ (11.0 mg/g), was observed from fermentation using two-stage pH control strategy when compared with other experiments using shake flasks (without pH control) and one-stage pH control strategy as depicted in Table 3. Though the 2-L stirred tank bioreactor with adequate monitoring of the culture conditions was used in fermentations without the control of the pH and with one-stage pH control strategy, the performance of the kinetics towards the biovanillin production was still not efficient when compared with that of two-stage control strategy (Table 3). Therefore, this shows that the method adopted by using the 2-L stirred tank bioreactor has been observed to enhance the performance of the fermentation kinetics towards the biovanillin production via application of proper control of the culture pH. Since, the kinetic performance for the biovanillin production was best determined when the culture pH has not been controlled at growth phase, though it was controlled at 6.0 during production phase of the fermentation processes (two-phase pH control strategy), as shown in Table 3. The improvements could be due to the ability of the fungus to adjust the pH of their culture conditions naturally during the growth phase, which enhanced their growth and metabolic activities, as well as facilitates the secretion of pertinent enzymes [16], in preparation to biovanillin bioconversion at the production phase by proper control of the culture pH at 6.0 accordingly. Also, the enhancement in the concentration of cell biomass observed in the fermentation with the two-stage pH control strategy could be because of high amount of glucose consumed (99.96%) by the fungus during the fermentation processes.

The lower time (12.6 h) that was observed by the fungus to double their number as well as the high value of the specific growth rate (0.055 h^{-1}) from the two-phase pH control strategy had equally confirmed that controlling the culture pH during the active growth phase of the fungus was not favorable to the fermentation processes for the biovanillin production by *Phanerochaete chrysosporium* ATCC 24725 when compared with the method in which the whole fermentation processes was controlled at pH 6.0, as presented in Table 3.

Table 3 Comparison for the performance of pH control strategy toward biovanillin production

Kinetic factors	Shake flask (pH 6.0)	Without control (initial pH 6.0)	One-phase control (pH 6.0)	Two-phase control
Maximum production, P_{Max} (mg/L)	93	98	124	131
Initial ferulic acid concentration (mg/L)	501	504	502	501
Final ferulic acid concentration (mg/L)	130	47	21	13
Ferulic acid converted to biovanillin (%)	25	21	26	27
Molar yield (%)	24	25	32	33
Initial glucose concentration (mg/L)	3120	24,990	24,860	24,860
Final glucose concentration (mg/L)	80	550	180	10
Glucose consumed (%)	94.87	97.80	99.28	99.96
X_{max} concentration (g/L)	5.60	11.14	12.14	12.52
Specific growth rate, μ (h^{-1})	2.6×10^{-2}	5.4×10^{-2}	5.3×10^{-2}	5.5×10^{-2}
Max Specific Growth rate, μmax (h^{-1})	5.2×10^{-2}	0.108	0.106	0.110
Yield coefficient of biomass to glucose, $Y_{x/s}$ (mg/g)	67.0	467	499	503
Yield coefficient of biovanillin to glucose, $Y_{p/s}$ (mg/g)	2.80	4.10	5.10	5.30
Yield coefficient of biovanillin to biomass, $Y_{p/x}$ (mg/g)	4.30	9.10	10.8	11.0
Maximum productivity (mg/L)	1.30	1.20	1.60	1.80
Overall productivity (mg/L/h)	0.11	0.67	1.22	10.8
Doubling time, td (h)	26.7	12.8	13	12.6
t (h)*	72	84	78	72

*Time at which the biovanillin production reached maximum

4 Conclusion

Biovanillin conversion from lemongrass leaves hydrolysates was performed using *Phanerochaete chrysosporium* ATCC 24725 in a 2-L stirred tank bioreactor using various control strategies. The fermentation performance using single control strategy by controlling culture pH at 6.0 revealed the highest biovanillin production (124 mg/L) with 32% molar yield. Improvement from the biovanillin production (131 mg/L) was observed from the two-stage with pH controlled at 6.0 during the production phase. Therefore, proper application of pH control during fermentation would not only provide an enabling culture conditions to support the growth of the fungus but also facilitate the release of pertinent enzymes that are involved into the biovanillin biotransformation. The research further suggests that other significant parameters like temperature and stirring speed could be optimized to investigate their influence towards the improvement of the biovanillin production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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