J Ind Microbiol Biotechnol (2014) 41:1563–1570
DOI 10.1007/s10295-014-1492-6

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Society for Industrial Microbiology and Biotechnology

High-yield recombinant xylanase production by *Aspergillus nidulans* under pyridoxine limitation

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Received: 17 April 2014 / Accepted: 12 July 2014 / Published online: 2 August 2014

Abstract The present study investigated the limitation of pyridoxine on an *Aspergillus nidulans* culture that produces xylanase B (XynB) as a client enzyme and was unable to synthesize pyridoxine. This technique was used to limit cell growth and divert substrate to product formation for a surface grown culture that could be used in trickle bed reactors. It was observed that growth was limited when pyridoxine was absent, while enzyme production was unaffected. Enzyme production was 1,026 U after 480 h of continuous fermentation, which was similar to a culture that grew on medium with pyridoxine. Furthermore, the present study investigated the growth rate of *A. nidulans* with pyridoxine in the medium and determined the productivity of XynB production with and without pyridoxine. A maximum growth rate of 0.311/h was observed. The maximum XynB productivity of 21.14 U/g h was achieved when pyridoxine was not added to the medium.

Keywords Enzymes · Hydrolysis · Continuous production · Filamentous fungi · Pyridoxine limitation · Xylanase

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Introduction

Industrial scale enzyme production with filamentous fungi mainly uses two fermentation processes: solid state (SSF) or submerged fermentation (SmF). No other process has proven to be applicable due to the difficulty of culturing filamentous fungi. Filamentous fungi grow as a mycelium, which can lead to clogging of pipelines due to their affinity for surfaces.

Most biotechnological processes are used for the production of metabolites (e.g. organic acids, alcohols, extracellular proteins, etc.). The growth of the organism and, therefore, the accumulation of biomass, is often not the priority. For these processes, cell mass that is produced can be considered a byproduct. The formation of cell mass uses carbon and energy compounds that could potentially be used for product formation. In many studies it was proposed to limit carbon and energy supply in chemostats to a level where the supplied energy is equal to the maintenance energy of the organism [13, 14]. Then, the organism will approach a zero growth rate when the energy consumed is near the maintenance ratio [10]. It was shown that submerged filamentous fungi cultures conidiate when nitrogen and carbon are limited [4, 5, 9]. Previous studies used retentostat cultures to analyze product formation under carbon and energy limited conditions. To achieve a retentostat, a chemostat was equipped with a filter with a pore size smaller than the size of the organism (0.2 μm), which trapped cells in the reactor. After the desired cell mass concentration was reached, the continuously supplied medium had limited carbon and energy content to reduce the metabolic rate. A previous study reported a growth rate of *Aspergillus niger* approaching zero after 6 days when grown in a retentostat under carbon and energy limited conditions [6]. However, the growth yield was constant,

leading to an increase in cell mass over time. An interesting observation from the study was how the percent of respired carbon changed over time [6]. With decreasing specific growth rate, the percent carbon respired increased. Conidiation, which is asexual reproduction, was observed after 2 days and melanin rapidly increased after 4 days. Stress on the cells, initiated by carbon and energy limitation, induces the transcription of *brlA* [16]. The transcription factor *brlA* is a positive regulator for conidiation [1, 11]. From the results presented in previous studies, [2, 6, 13, 14, 19], it is not conclusive if continuous product formation with zero growth rate over a prolonged period of time using carbon and energy limitation is feasible. The studies showed that over the test period the cell mass concentration increased until the end of the experiment. Additionally, no literature on zero growth rate experiments was found with filamentous fungi grown on surfaces. Furthermore, no literature was found initiating a zero growth rate by limiting essential nutrients in the medium that do not supply the organism with carbon or energy. Examples of such nutrients are vitamins and coenzymes necessary for cell functions.

Reducing growth rate can maximize product formation since substrate would be used for product formation and not for vegetative growth. A compound present in many fungal media that is necessary for fungal growth is pyridoxine. Pyridoxine is a vitamin and precursor for pyridoxal-5′-phosphate (PLP), which is involved in many reactions in amino acid metabolism. One reaction is transamination, where an α-amino acid is converted to an α-keto acid [20]. PLP acts as a coenzyme together with the enzyme in the form of a Schiff base. It is also involved throughout amino acid biosynthesis in the conversion of aspartate to lysine, threonine to methionine and α-ketoisocaproate to leucine. PLP is a trace element and is only needed in very low concentrations. This study proposed that a limitation of pyridoxine in the fermentation medium for an *Aspergillus nidulans* strain with a pyridoxine deficiency marker would lead to a shift of substrates from cell growth to protein formation. No literature has been found where the limitation of pyridoxine was tested on filamentous fungi. Immobilized cell systems, such as trickle bed reactor applications, could benefit from this approach since growth related issues, such as clogging and fouling, often occur in such systems and must be controlled.

Materials and methods

Strain and spore formation

A recombinant *A. nidulans* A773 (gentotype, *pyrG89*; *wA3*; *pyroA4*) obtained from the Fungal Genetic Stock Center (Kansas City, MO, USA) was used to conduct the experiment. The strain has a pyridoxine (*pyroA4*) marker and a genetic modification at the *pyrG* locus that expresses and secretes Xylanase B (from *Penicillium funiculosum*) as a client protein [15]. Spores were kept in a 20 % glycerol, 10 % lactose solution as a stock culture at −80 °C. Spores were made by using a solid medium containing 10.0 g/l glucose, 50 ml/l 20× Clutterbuck salt solution (120 g/l $NaNO_3$, 10.4 g/l KCl, 10.4 g/l $MgSO_4$, 30.4 g/l KH_2PO_4), 1 ml/l 1,000× trace element solution (22 g/l $ZnSO_4{\cdot}7H_20$, 11 g/l H_3BO_3, 5.0 g/l $MnCl_2{\cdot}7H_2O$, 5.0 g/l $FeSO_4{\cdot}7H_2O$, 1.6 g/l $CoCl_2{\cdot}5H_2O$, 1.6 g/l $CuSO_4{\cdot}5H_2O$, 1.1 g/l $Na_2MoO_4{\cdot}4H_2O$, 50 g/l Na_2-EDTA), 1 ml of a 1 g/l pyridoxine solution, and 1.5 % agar. The pH was adjusted to 6.5 using 10 M NaOH. Twenty microliter of stock spore solution were distributed on the agar surface. The plates were kept for 48 h at 37 °C. After incubation the plates were stored at 4 °C until usage.

Fermentation medium

The fermentation medium contained 47.6 g/l maltose and 10.0 g/l glucose as carbon sources. Maltose is necessary to activate the promoter for Xylanase B expression. The medium also contained 50 ml/l 20× Clutterbuck salt solution, 1 ml/l 1,000× trace element solution, and 1 ml of a 1 g/l pyridoxine solution. For the maximum growth rate experiment, maltose and glucose concentrations were varied, but were kept at the same maltose:glucose ratio (4.7:1). The medium pH was adjusted to 6.5 using 10 M NaOH. Medium was sterilized by autoclaving at 121 °C for 20 min.

Determination of maximum growth rate of *A. nidulans*

All experiments were conducted in Petri dishes with surface grown cultures and not in submerged flask cultures. The results obtained with surface grown cultures represented more realistically the conditions of immobilized cell systems, such as in a trickle bed reactor. Before the maximum growth rate (μ_{max}) could be obtained, multiple experiments were necessary to investigate the specific growth rate (μ_{net}) at different initial substrate (S) concentrations. The Monod model was used as the underlying base for μ_{max} and μ_{net} determination. It was required to measure cell mass concentration over time to calculate μ_{net}. Since the organism is a filamentous fungus, representative sampling was not possible. To overcome this problem, multiple parallel tests were started with different inoculation times. The tests were carried out in Petri dishes. Each dish was filled with 20 ml of medium with identical substrate concentration. All parallel tests were inoculated with the same amount of spore solution (20 µl) with the same spore concentration. Two dishes were inoculated for each time interval of 12, 24, 36 and 48 h. After these time intervals, the

complete cell mycelium was harvested, washed with deionized water and dried at 50 °C for 1 day. The protein concentration in the broth was analyzed by the Bradford assay [3]. An aliquot of broth was used for sugar analysis with HPLC as described below. The logarithm of dry cell mass was plotted vs. time. The slope of the regression line in the linear region of the plot represented μ_{net}. This process was repeated for different initial maltose and glucose concentrations. The obtained μ_{net} was plotted in a reciprocal form vs. $1/S$. For this plot, S represented glucose concentration plus the glucose equivalent of maltose. A linear regression of the data was used to calculate μ_{max} and K_S.

Evaluation of XynB production with *A. nidulans* under pyridoxine limitation

Two tests with different transplanting frequencies, 24 and 48 h, were chosen. To grow a mycelium, a Petri dish was filled with 20 ml of medium and inoculated with 20 µl of spore solution. The culture was allowed to grow at 37 °C until a mycelium covered the surface of the medium. After inoculation, part of the mycelium was manually transferred into a Petri dish previously filled with medium lacking pyridoxine. This culture was allowed to grow either 24 or 48 h and then was transferred again into medium without pyridoxine. This was repeated until protein production decreased. Then, the mycelium previously grown on pyridoxine-free medium was transferred into medium with pyridoxine, and after either 24 or 48 h it was again transferred into medium without pyridoxine. The cultures were transferred five and nine times for the 24 and 48 h cultures, respectively. After each transfer interval, protein concentration in the medium was measured by the Bradford assay, xylanase activity was determined with DNS reagent, the wet mass of transferred mycelium was measured, the pH was measured and the sugar and organic acid concentration were measured with HPLC. Additionally, a control mycelium, which was always placed into medium with pyridoxine, was evaluated. Both experimental treatments and the control were evaluated in duplicate.

Determination of *A. nidulans* XynB productivity under pyridoxine limitation

In order to obtain the enzyme productivity under pyridoxine limitation, a mycelium was grown on medium with pyridoxine and then transferred into medium without pyridoxine. A Petri dish was filled with 20 ml medium with pyridoxine and was inoculated with 20 µl of spore solution. The culture was grown for 48 h at 37 °C. After 48 h, the culture was transferred into medium without pyridoxine and after 48 h more it was again transferred into medium without pyridoxine. After this second transfer, mycelium growth was examined based on visual comparison before and after incubation. After 24 h after the second transfer, a sample of the liquid medium was taken and analyzed for sugar concentration with HPLC, protein concentration with Bradford assay and xylanase activity with DNS reagent. The mycelium was washed with DI water, dried for 1 day at 50 °C and dry cell mass was measured. Enzyme productivity was determined by calculating the total activity (U) from the volumetric activity (U/ml) and dividing it by the total dry cell mass (g) and 24 h. The enzyme activity was measured to determine the existence of XynB. The test was performed in triplicate and compared to a control culture that grew on medium with pyridoxine.

Determination of protein concentration

Protein concentration was determined using a modified Bradford assay [7]. Forty µl of Bradford coommassie solution was pipetted into a well of a 96-well plate. In order to stay within the absorbance calibration curve limit of 2.5 at 575 nm, the amount of enzyme solution varied depending on the protein concentration received from the test. DI water was added to achieve a total volume of 200 µl. The blank well contained only 40 µl Bradford solution and 160 µl DI water. The absorption was measured using a UV–Vis 96-well plate reader (Tecan Infinite M200, Männedorf, Switzerland) at 595 nm.

Determination of XynB activity

XynB was analyzed for its specific activity on xylan from beechwood (Sigma Aldrich). Forty-nine µl of 0.5 % xylan solution prepared with 25 mM ammonium acetate buffer at pH 6 and 1 µl of enzyme solution were added into a well of a 96-well plate. The plate was incubated in a water bath at 50 °C for 15 min. After incubation, the 96-well plate was removed from the water bath and 50 µl of dinitrosalicylic acid (DNS) reagent was added immediately to terminate enzymatic hydrolysis [8]. The 96-well plate was placed in a second water bath at a temperature of 100 °C for 5 min to achieve color formation. After the reaction time, 100 µl of the liquid was transferred in a 96-well reading plate and analyzed at 575 nm for reducing sugar concentration. The specific activity was calculated based on the spectrophotometer values with the following equation:

$$U = \left(((A - 0.047)/F) \times \left(\frac{V_{assay}}{t} \right) \right) / P_{enzyme} \qquad (5.1)$$

where U is the specific activity [µmol of reducing sugar/mg protein min], A absorbance at 575 nm, F calibration factor, V_{assay} assay volume (200 µl), t incubation time [min] and p_{enzyme} mass protein in enzyme used [mg].

The final results were given in XynB activity units per volume, U_V.

Table 1 Total cell mass, protein concentration, substrate concentration (glucose + glucose equivalent from maltose), specific growth rate and yields for *Aspergillus nidulans* at different initial substrate concentrations, with standard deviation of two replicates

Initial substrate concentration (g/l)	Total cell mass after 48 h (mg)	Protein concentration after 48 h (g/l)	Substrate concentration after 48 h (g/l)	μ_{net} (per hour)	$Y_{X/S}$ (g cells/g substrate)	$Y_{P/X}$ (g protein/g cells)	$Y_{P/S}$ (g protein/g substrate)
59.3	119 ± 11.3	0.019 ± 3.54E-5	52.2 ± 0.77	0.061	0.834 ± 0.02	0.0048 ± 9.1E-5	0.0027 ± 0.0003
29.7	93 ± 9.90	0.009 ± 0.002	20.0 ± 3.43	0.063	0.484 ± 0.10	0.0025 ± 7.1E-5	0.0010 ± 0.0008
11.9	79 ± 2.83	0.018 ± 0.002	1.73 ± 0.31	0.095	0.390 ± 0.002	0.0054 ± 0.0003	0.0017 ± 9.4E-5
5.93	57 ± 2.12	0.011 ± 0.0005	0.20 ± 0.02	0.194	0.493 ± 0.017	0.0058 ± 0.0007	0.0020 ± 0.0001
3.00	28 ± 2.12	0.010 ± 0.001	0	0.214	0.464 ± 0.012	0.0187 ± 0.0053	0.0034 ± 0.0003
1.19	14 ± 0.71	0.004 ± 0.0007	0	0.146	0.611 ± 0.030	0.0025 ± 0.0007	0.0017 ± 0.0007

$$U_V = U \cdot C_P \tag{5.2}$$

where C_P is the protein concentration in mg/ml.

Sugar and organic acid analysis

Concentrations of glucose and maltose were analyzed on an HPX-87P column (Bio-Rad, Sunnyvale, CA, USA). The eluent was HPLC grade DI-water with a flow rate of 0.6 ml/min at 85 °C and a refractive index detector (1100 Series Agilent, Santa Clara, CA, USA) [17]. Succinic acid and acetic acid were analyzed on a HPX-87H column (Bio-Rad, Sunnyvale, CA, USA). The eluent was 0.005 M sulfuric acid with a flow rate of 0.6 ml/min at 60 °C. A refractive index detector (1100 Series Agilent, Santa Clara, CA, USA) was used for detection [17].

Results and discussion

Growth rate of *A. nidulans*

Most studies that deal with analyzing growth kinetics of filamentous fungi use either submerged cultures or cultures grown on solid medium or surfaces. When grown as a surface culture, the parameters for growth rate are based on circumferential growth, rather than on a mass per volume unit. The present study used a surface grown culture, but applied the same methodology as for submerged cultures. Table 1 shows the total cell mass, protein and substrate concentrations after 48 h, as well as the specific growth rates for the corresponding initial substrate concentrations and yields. Using parallel tests to emulate sampling at different time points worked well for this system and the growth curves showed the familiar pattern observed for many microorganisms. The total cell mass after 48 h increased with increasing initial substrate concentration. However, it was found that μ_{net} does not increase with substrate concentration. In fact, substrate inhibition as determined by a double reciprocal plot of μ_{net} versus S was observed (data not shown). Hence, the fungus grew at a slower rate at higher substrate concentrations.

The highest μ_{net} with a value of 0.214/h was found with initial substrate concentrations of 2.4 g/l maltose and 0.45 g/l glucose. At higher substrate concentrations, the fungus grew more slowly, but continuously for at least 48 h. At lower substrate concentrations, the fungus grew fast to exploit all nutrients, but stopped growing when nutrients were depleted. The maximum specific growth rate was 0.311/h, and K_S was 1.33 g/l.

It has been found that mycelia of filamentous fungi develop differently for different substrate concentrations in the environment. At high substrate concentration, hyphae form a dense hyphal network with more branches per area to utilize nutrients more efficiently in that area. When substrate concentration is low, hyphae form fewer branches per area and grow longer in order to reach a location with higher substrate concentration [12]. This behavior was also observed in the present study. Cultures with low initial substrate concentration were more like a gel throughout the liquid; whereas, cultures with high initial substrate concentration developed a mycelium layer on top of the surface of the liquid at the end of the test.

A growth rate based on mycelium mass per time is more useful for industrial applications than a growth rate based on circumferential growth. It is often not practical to measure circumferential growth in technical applications. Cultures that grow in nutrient rich medium also become thicker (more mass per area), which is not be included in measuring circumferential growth.

XynB production with inhibited growth

It was observed that the fungus produced the desired enzyme, XynB, even when growth was suppressed. Figure 1 shows the total cumulative XynB activity over time for the experiment with a transplanting frequency of 48 h. The average XynB activity was 10.0 ± 5.13 U/ml (mean ± one standard deviation) for the culture grown without pyridoxine. The control showed an average XynB

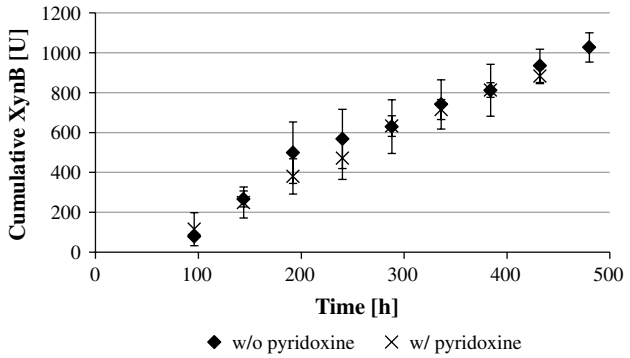

Fig. 1 XynB production over time with limited pyridoxine and 48 h transplanting frequency. The culture was initially (0–48 h) grown on medium with pyridoxine and again at 288 h. *Error bars* represent standard deviation of two replicates

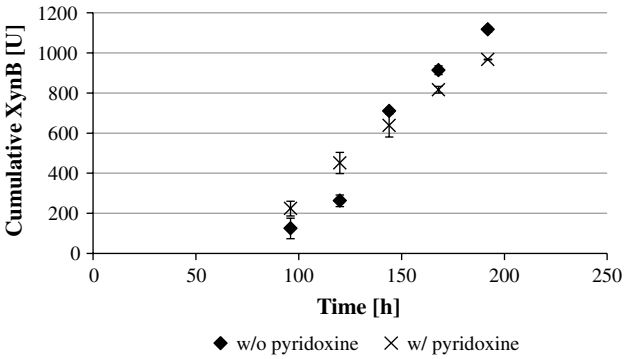

Fig. 2 XynB production over time with limited pyridoxine and 24 h transplanting frequency. The culture was initially (0–72 h) grown on medium with pyridoxine and again at 144 h. *Error bars* represent standard deviation of two replicates

activity of 9.78 ± 3.0 U/ml. It was observed that the protein concentration dropped to 0.08 g/l after 192 h when grown on medium without pyridoxine. The 240 and 288 h samples showed a yellow colored broth that smelled similar to urine. Medium pH dropped from 8.5 to 4.5 in these samples. The culture was transferred after 288 h into medium with pyridoxine to recover. At 336 h, no smell was detected and the broth color was the normal amber color. However, even though protein concentrations in 240 h and 288 h samples were lower than previous samples, the XynB activities were comparable. This observation indicated a higher proportion of XynB in the total protein mix in the 240 and 288 h samples than in previous samples. No difference in XynB production was observed between the test without pyridoxine and the control. The cell matrix without pyridoxine had enough PLP stored to perform basic reactions and protein formation without growth.

With a transplanting frequency of 24 h, the average XynB activity was 9.63 ± 5.64 and 9.88 ± 2.06 U/ml for the culture grown without and with pyridoxine, respectively. Figure 1 shows the cumulative XynB activity over time. The phenomenon of decreased protein formation with simultaneous increase in activity, which was found for both transplanting frequencies, showed that XynB formation is independent of mycelium growth. Furthermore, it can be seen in Fig. 2 that when the culture was transplanted with a 24 h frequency, the cumulative XynB production reaches the same levels as the culture with 48 h transplanting frequency, but at 192 h as opposed to 480 h. Hence, it is not necessary to use a 48 h frequency since the same XynB activity is already reached after 24 h.

Substrate utilization is shown in Fig. 3. Maltose was completely utilized at all times with 48 h transplanting frequency. Glucose was completely utilized at all times except 240 and 288 h when grown on medium without pyridoxine. The glucose concentration increased to 12.99 g/l at

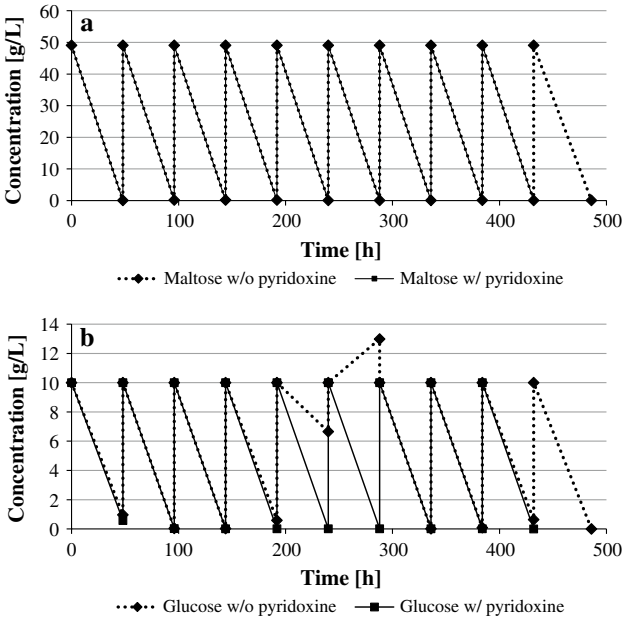

Fig. 3 Maltose (**a**) and glucose (**b**) utilization during enzyme production with limited pyridoxine with 48 h transplanting frequency

288 h, indicating that the metabolism still utilizes all maltose and converts some of it to glucose, which is then not used further. With 24 h transplanting frequency, the mean residual maltose and glucose concentrations were 11.39 and 4.98 g/l, respectively, when grown on medium without pyridoxine (Fig. 4). The maltose and glucose utilizations were higher for the culture grown on pyridoxine than for the culture grown on medium without pyridoxine. The control showed mean residual maltose and glucose concentrations of 5.30 and 2.03 g/l, respectively. Compared to the culture grown without pyridoxine, the control showed new mycelium growth, which required substrate and explained the faster nutrient utilization.

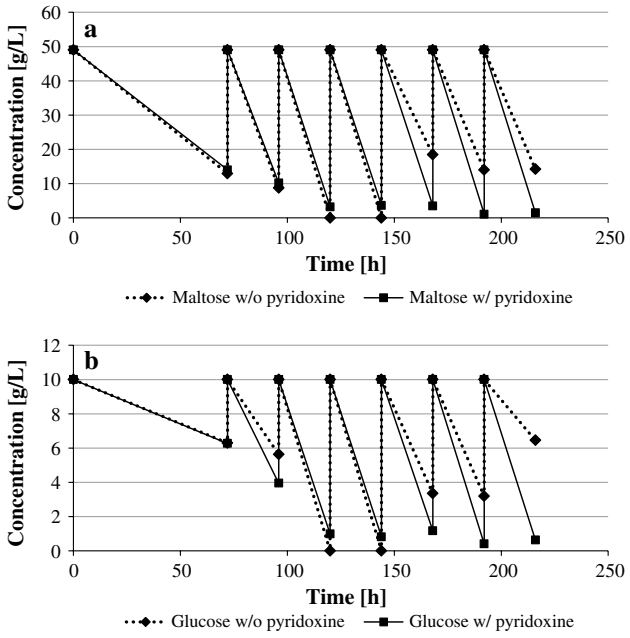

Fig. 4 Maltose (**a**) and glucose (**b**) utilization during enzyme production with limited pyridoxine with 24 h transplanting frequency

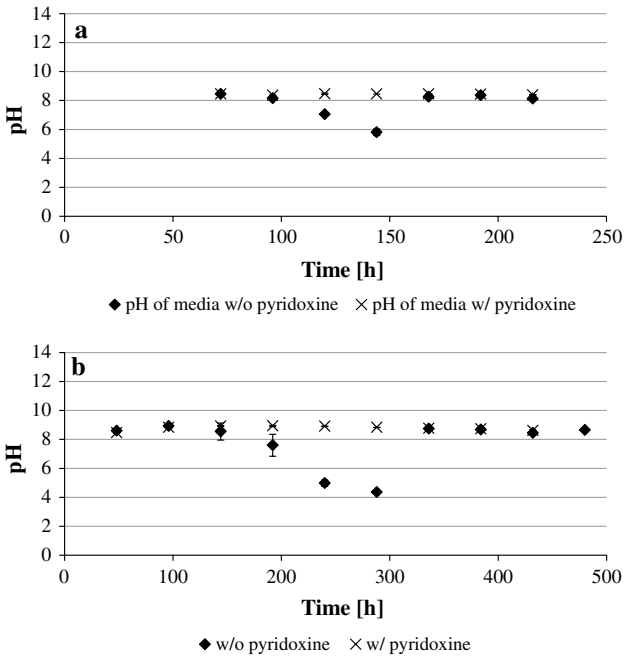

Fig. 5 pH development over time for 24 h (**a**) and 48 h (**b**) transplanting frequency. *Error bars* represent standard deviation of two replicates

After 192 h pyridoxine needed to be replenished for the culture inoculated without pyridoxine for further enzyme production. The moment of pyridoxine replenishment can be monitored by measuring pH. It is recommended to replenish pyridoxine before pH drops below 6 in order to avoid a decrease in protein production (Fig. 5). The positive correlation of pH to protein production is a useful tool for reactor control and monitoring since online monitoring of pH is ready available, while online measurement of protein concentration is more difficult.

No measurement of cell mass was performed due to the impossibility of taking homogenous samples. Pictures were taken to document the surface area of the fungus throughout the experiment and to evaluate whether the mycelia surface area increased. No visual growth of surface area (enlargement of mycelia) took place when the fungus was kept on medium without pyridoxine. The fungus's color became darker over time while using medium without pyridoxine, which indicated melanin formation as in [6].

The cause for the pH decrease over time under pyridoxine limitation is not known, but it was ties to two observations. First, an increase in succinic acid concentration from 0.06 to 0.26 g/l could be observed when pH decreased between 240 and 288 h. Succinic acid concentration decreased back to 0.08 g/l at 336 h (data not shown). Acetic acid was also measured and was not observed to increased. It was expected that pyridoxine limitation caused an increase in organic acids and/or amino acids due to the involvement of pyridoxine in amino acid metabolism. Other organic acids and amino acids were not analyzed with HPLC. Second, as noted above there was a strong odor similar to urine that was present when the pH decrease was observed. A previous study using methionine-requiring *Sacchromyces cerevisiae and Sacchromyces carlsbergensis* mutants observed that hydrogen sulfide was produced when the medium was deficient in pyridoxine [21]. The H_2S was formed from reduction of sulfate in sulfate salts from the medium. Though H_2S was not analyzed for in this study, the combination of a foul odor with a drop in pH could indicate the production of H_2S.

Besides the changes that occur in the metabolism based on pyridoxine limitation, fungal autolysis could also be responsible for pH decrease [18]. Lack of pyridoxine indirectly leads to starvation by disturbing amino acid metabolism, which has been shown to initiate autolysis [22]. Autolysis is a process in which the fungus enzymatically breaks down old fungal mass in order to utilize the nutrients for fungal hyphae tip growth. This breakdown of fungal mass could release compounds responsible for pH decrease.

Table 2 shows all kinetic factors obtained from this experiment. The goal was to determine protein productivity of the *A. nidulans* culture when initially grown on medium with pyridoxine and then transferred onto medium without pyridoxine. The results presented are obtained after transplanting the culture a second time. After the second transplantation, no visual growth was observed with the culture without pyridoxine. The productivity was 21.14 U/g dry

Table 2 Factors obtained of productivity experiment

Factors	w/o pyridoxine	w/pyridoxine
Initial maltose (g/l)	47.62	47.62
Initial glucose (g/l)	10.00	10.00
Initial total substrate (g/l)	60.28	60.28
Final maltose after 24 h (g/l)	7.68 ± 1.30	6.02 ± 0.993
Final glucose after 24 h (g/l)	8.56 ± 1.90	4.92 ± 0.397
Final total substrate after 24 h (g/l)	16.67	11.28 ± 1.40
XynB concentration after 24 h (U/ml)	17.32 ± 3.15	11.27 ± 4.38
Dry cell mass (g)	0.68 ± 0.13	0.78 ± 0.08
$Y_{P/X}$ (U/g)	507.42 ± 37.54	194.21 ± 52.81
$Y_{P/S}$ (U/g)	21.84 ± 8.57	13.95 ± 5.64
Productivity (U/g h)	21.14 ± 1.56	8.09 ± 2.20

Total substrate = $C_{glucose} + C_{maltose}/0.947$. $Y_{P/X}$ protein to dry cell mass yield, $Y_{P/S}$ protein to substrate yield

cell mass h for the culture without pyridoxine and 8.09 U/g dry cell mass h for the culture with pyridoxine. The productivity without pyridoxine was 2.6 times greater than the productivity with pyridoxine over the same time period, which was also represented in the yields. The protein to dry cell mass yields ($Y_{P/X}$) of the culture without pyridoxine and the culture with pyridoxine were 507.42 and 194.21 U/g, respectively. The protein to substrate yield ($Y_{P/S}$) was higher for the culture with pyridoxine, 21.84 U/g, than the culture without pyridoxine, 13.95 U/g.

The greater yields and productivities, similar enzyme activity concentrations and lack of growth for the cultures grown in pyridoxine-deficient media compared to cultures grown with pyridoxine show that using an *A. nidulans* mutant auxotrophic for pyridoxine with a pyridoxine-deficient media diverted substrate from cell growth to enzyme production without any detrimental effect of enzyme production. The lack of cell growth suggests that using a fungal strain auxotrophic for a cofactor or coenzyme that is not incorporated into the desired product can be an effective strategy for limiting growth in reactors that can suffer issues from excessive cell growth, such as a trickle bed reactor. The technique demonstrated in this study would allow the use of a trickle bed reactor for continuous enzyme production using liquid media, which combines the ease of control and continuous operation of a SmF and the preservation of the mycelia structure of filamentous fungi found in SSF.

Conclusions

It was successfully demonstrated that a fungus mutant requiring pyridoxine for growth could maintain enzyme production even when growth is limited by limiting pyridoxine. However, the continuous enzyme production cannot go on forever. Pyridoxine needed to be replenished intermittently. Since pH correlates with pyridoxine depletion, monitoring pH allowed identification of the correct moment when pyridoxine needed to be added. Pyridoxine limitation caused stress on the cell, observed by a change of the medium color to dark brown, which indicates conidiation and melanin formation. The increased stress on the cells due to pyridoxine limitation was observed to have no effect on XynB formation.

Acknowledgments The authors would like to acknowledge the USDA National Research Initiative Biobased Products and Bioenergy Research Program and the Oklahoma Agricultural Research Station for providing financial support for this work.

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