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Facilitation of L-Lactic Acid Fermentation by Lignocellulose Biomass Rich in Vitamin B Compounds

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S *Supporting Information*

ABSTRACT: Vitamins are important nutrients for many fermentations, but they are generally costly. Agricultural lignocellulose biomass contains considerable amounts of vitamin B compounds, but these water-soluble vitamins are easily lost into wastewater discharge during pretreatment or detoxification of lignocellulose in biorefinery processes. Here, we showed that the dry acid pretreatment and biodetoxification process allowed the preservation of significant amounts of vitamin B, which promoted L-lactic acid fermentation efficiency significantly. Supplementation with specific vitamin B compounds, VB_3 and VB_5, into corn stover hydrolysate led to further increases of cellulosic L-lactic acid yield and fermentation rates. This study provided a new solution for the enhancement of biorefinery fermentation efficiency by using vitamin B compounds in lignocellulose biomass.

KEYWORDS: *vitamin B, L-lactic acid, lignocellulose biomass, dry acid pretreatment, biodetoxification*

INTRODUCTION

Lignocellulose biomass is the preferred feedstock option for future production of large commodity biofuels and biobased materials such as biodegradable poly lactic acid (PLA).[1] Biorefinery processes typically include pretreatment, detoxification, saccharification, and fermentation.[2] The main focus of the biorefinery field is to overcome technical barriers, such as the disruption of lignocellulose structure for increasing cellulose accessibility,[2,3] the removal of inhibitors to allow the sugars to be fermented,[4] saccharification at high solids loading and viscosity,[5] and cofermentation of glucose and the pentoses derived from hemicellulose.[6,7] In addition to cellulose, hemicellulose, and lignin, lignocellulose biomass contains a range of other chemical substances, the function of which is to stimulate growth and metabolism of plant cells.[8,9] Surprisingly, few studies have focused on the importance of these compounds (e.g., micronutrients or vitamins) in the biorefining process.

In a previous study, we showed that agricultural lignocellulose biomass, such as corn stover, wheat straw, and rice straw, contain certain amounts of vitamin B compounds after harvest, sun-drying, collection, storage, and even harsh pretreatment operations.[10] The presence of vitamin B compounds has not been fully acknowledged in conventional lignocellulose biorefinery processing, because the overload of water normally washes out most of the water-soluble components, including vitamin B, from the feedstock.[11−13] For example, in conventional dilute acid pretreatment at low solids contents (5−20%, w/w), vitamins are removed from lignocellulose biomass into the prehydrolysate at liquid phase.[2,11,14] In dilute alkaline, organosolv, or ionic liquid pretreatments, the washing step is performed for detoxification of the pretreated feedstock, and most of the vitamin B compounds in lignocellulose biomass are washed into the wastewater stream.[13−15] In contrast, no prehydrolysate or wastewater stream is generated in our newly developed dry acid pretreatment and biodetoxification (DryPB) technology.[6] Therefore, the undegraded vitamin B compounds are still present in the feedstock, and a saccharification slurry with high amounts of vitamin B compounds can be generated. One of the vitamin B compounds, biotin (VB_7), was found to play a key role in cellulosic glutamic acid production by *Corynebacterium glutamicum* S9114,[10] but the effects of vitamin B compounds in lignocellulose feedstock on other kinds of fermentation have not yet been investigated.

Vitamin B compounds are the constituents or precursors of coenzymes and play roles in energy metabolism, protein metabolism, and carbohydrate and fatty acid metabolism, among others.[9,16] L-Lactic acid, the monomer of biodegradable poly lactic acid (PLA),[17] is known to require supplementation of many nutrients, including vitamin B compounds, in its fermentation process[7,16] because most lactic acid bacteria are auxotrophic for many amino acids and vitamins.[16,18−22] In the current study, we investigate how vitamin B compounds from lignocellulose biomass promote L-lactic acid fermentation by *Pediococcus acidilactici* ZY271. The requirement of vitamin B compounds in L-lactic acid fermentation in synthetic medium (prepared by using sugars, salts, amino acids, vitamins, and nucleic acids, as discussed in the Strains and Media section) was compared with that in corn stover hydrolysate (CSH), and it was found that the vitamin B compounds present in corn stover could satisfy some of the requirements in L-lactic acid fermentation. With specific supplementation of vitamins B_3 and B_5, the L-lactic acid productivity and yield were increased to levels comparable to those obtained with nutrient-rich

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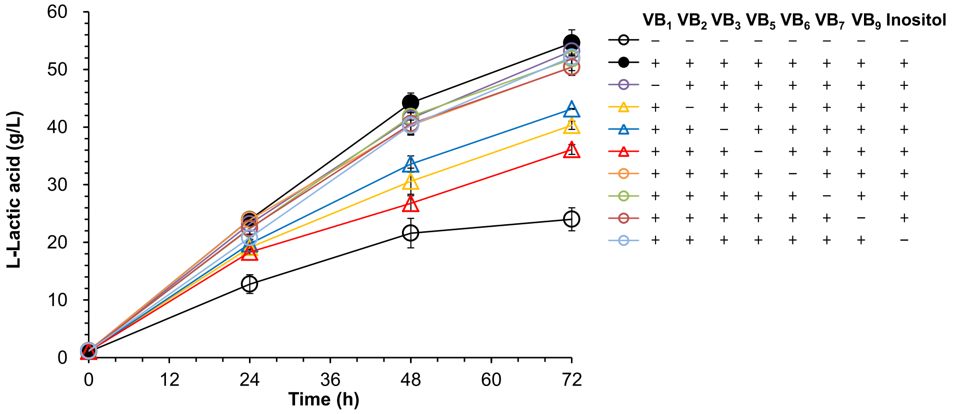

Figure 1. L-Lactic acid fermentation in synthetic medium with vitamin B deficiency. The detailed composition of the synthetic medium is described in the Strains and Media section. Vitamin B compounds were supplemented as follows: 1070 μg/L thiamine·HCl (VB_1), 495 μg/L riboflavin (VB_2), 9840 μg/L niacin (VB_3), 2370 μg/L calcium pantothenate (VB_5), 939 μg/L pyridoxine·HCl (VB_6), 18.3 μg/L biotin (VB_7), 583 μg/L folic acid (VB_9), and 58.4 μg/L inositol. Fermentation was carried out in 100 mL flasks containing 20 mL of synthetic medium at 42 °C and 150 rpm. The experiments were carried out in duplicate, and the data used here was taken from the average of two parallel experiments.

medium. This study shows the promotion of biobased chemical fermentation through the use of vitamins in lignocellulose biomass and also gives an option for reducing media cost via reduced addition of costly exogenous vitamins in lignocellulose biorefineries.

■ MATERIALS AND METHODS

Raw Materials, Enzymes, and Reagents. Corn stover was harvested from Tongliao, China, in Fall 2016. The material was sun-dried and milled by being passed through 10 mm (diameter) apertures with a hammer crusher, and it contained 31.2% of cellulose, 22.3% of xylan, 20.8% of lignin, 6.2% of ash by weight percentage measured according to NREL protocol.[23]

Commercial cellulase Cellic CTec 2.0 was purchased from Novozymes; its filter paper activity was 203.2 FPU/mL, its cellobiase activity was 4900 CBU/mL, and its protein content was 87.3 mg/mL, as determined in our previous study.[12] Glucoamylase GA-L NEW was purchased from Genencor, and the enzymatic activity was 103 900 WU/mL.[12] Yeast extract that was used for component analysis as described in the literature[16] was from Merck KGaA. Yeast extract that was used for seed culture in this study was purchased from Oxoid Company. Amino acids and nucleic acids were purchased from Yuanju Biotech Company. Vitamin B compounds were purchased from Sinopharm Chemical Reagent Company. Other analytical-grade chemicals were purchased from Lingfeng Chemical Reagent Company.

Strains and Media. *Pediococcus acidilactici* ZY271 from the China General Microorganisms Collection Center (CGMCC 13611) in Beijing, China, was used for L-lactic acid production.[7] The synthetic fermentation medium was prepared according to the requirements for lactic acid fermentation by *Pediococcus acidilactici*. The ingredients included 55 g/L glucose, 17 g/L xylose, 2 g/L diammonium hydrogen citrate, 2 g/L sodium acetate, 0.3 g/L $MgSO_4$, 2 g/L K_2HPO_4, and 0.23 g/L $MnSO_4$. Amino acids, vitamins, and nucleic acids were added to yield a synthetic medium with a composition similar to that of simplified MRS medium containing 10 g/L yeast extract.[16] The supplements included (1) amino acids (250 mg/L glycine, valine, leucine, isoleucine, threonine, serine, asparagine, methionine, glutamic acid, phenylalanine, tyrosine, tryptophan, alanine, proline, aspartic acid, glutamine, lysine, histidine, arginine, and cysteine); (2) vitamins, including 1070 μg/L thiamine·HCl (VB_1), 495 μg/L riboflavin (VB_2), 9840 μg/L niacin (VB_3), 2370 μg/L calcium pantothenate (VB_5), 939 μg/L pyridoxine·HCl (VB_6), 18.3 μg/L biotin (VB_7), 583 μg/L folic acid (VB_9), and 58.4 μg/L inositol; and (3) nucleic acids (114.2 μg/L guanine hydrochloride and 143.1 μg/L adenine sulfate).

Pretreatment, Biodetoxification, and Corn Stover Hydrolysate Preparation. Dry acid pretreatment of corn stover feedstock was conducted according to Han and Bao.[11] The pretreated feedstock was biodetoxified using *Amorphotheca resinae* ZN1 (CGMCC 7452) to remove the inhibitors.[4] No liquid phase was generated or removed during the dry acid pretreatment and biodetoxification (DryPB) process (50% moisture). The corn stover hydrolysate (CSH) was prepared by enzymatic hydrolysis of the dry-acid-pretreated and biodetoxified corn stover at 15% (w/w) solids content with 10 mg of protein per gram of cellulose at 50 °C, pH 4.8, for 48 h. After removal of the solids, the prepared CSH contained 54.1 g/L glucose, 16.9 g/L xylose, and 1.1 g/L acetic acid with no furfural or HMF detected.

L-Lactic Acid Fermentation. One vial of the *P. acidilactici* ZY271 stock was inoculated into 20 mL of the simplified MRS medium (20 g/L glucose, 10 g/L yeast extract, 2 g/L diammonium hydrogen citrate, 5 g/L sodium acetate, 0.3 g/L $MgSO_4$, 2 g/L K_2HPO_4, and 0.23 g/L $MnSO_4$) and cultured at 42 °C and 150 rpm for 12 h; then, a 10% (v/v) inoculum size was used to inoculate fresh simplified MRS medium at 42 °C and 150 rpm for 5 h, according to Han et al.[12] The seed (10%, v/v) was used for lactic acid fermentation, which was conducted at 42 °C and 150 rpm in a 100 mL flask containing 20 mL of synthetic medium or CSH and 3 L fermentor (Biotech 3BG-4, Baoxing Company) containing 1 L of CSH. $CaCO_3$ (36 g/L) was added to neutralize the generated L-lactic acid, and 1% (v/v) glucoamylase solution was added to prevent cell flocculation. When the CSH was used as a substrate for fermentation, synthetic medium was added that lacked glucose, xylose, sodium acetate, $MgSO_4$, and K_2HPO_4, following the protocol of Han and Bao.[11]

Analytical Methods. Vitamin B compounds in the corn stover hydrolysate were assayed in the Shanghai Technical Center for Animal, Plant, and Food Inspection and Quarantine (http://shanghai.customs.gov.cn), Shanghai, China, and the assay methods were as follows. Thiamine (VB_1) was assayed with a fluorescence method via reverse-phase high-pressure liquid chromatography.[24,25] Riboflavin (VB_2) and folic acid (VB_9) were assayed on the basis of cell growth of a riboflavin and folic acid auxotrophic strain, *Lactobacillus rhamnosus* ATCC 7469.[25−27] Niacin (VB_3), pantothenate (VB_5), and biotin (VB_7) were assayed on the basis of cell growth of a niacin, pantothenate, and biotin auxotrophic strain, *Lactobacillus plantarum* ATCC 8014.[10,28,29] Pyridoxine (VB_6) was assayed on the basis of cell growth of a pyridoxine auxotrophic strain, *Saccharomyces carlsbergensis* ATCC 9080.[25] The principles of the above-mentioned microbiological assays for the vitamins were the same, and the procedures were similar. Here, we show the detailed procedures of the biotin (VB_7) assay as a typical case in Supporting Information Text S1.

Glucose, xylose, L-lactic acid, and inhibitors were measured using HPLC (LC-20AD pump, RID-10A refractive index detector,

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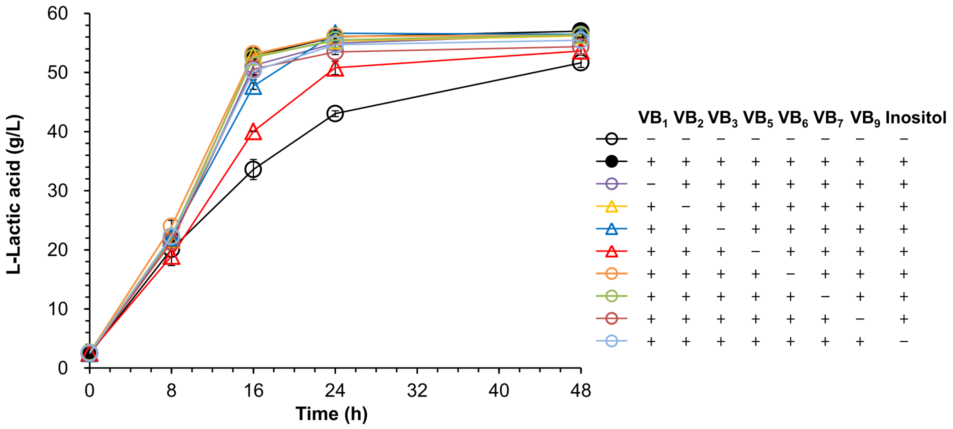


Figure 2. L-Lactic acid fermentation in corn stover hydrolysate with vitamin B deficiency. The prepared corn stover hydrolysate contained 265 μg/L thiamine (VB_1), 1420 μg/L riboflavin (VB_2), 738 μg/L niacin (VB_3), 30 μg/L pantothenate (VB_5), 63 μg/L pyridoxine (VB_6), 59 μg/L biotin (VB_7), 4 μg/L folic acid (VB_9). Vitamin B compounds were supplemented as follows: 1070 μg/L thiamine·HCl (VB_1), 495 μg/L riboflavin (VB_2), 9840 μg/L niacin (VB_3), 2370 μg/L calcium pantothenate (VB_5), 939 μg/L pyridoxine·HCl (VB_6), 18.3 μg/L biotin (VB_7), 583 μg/L folic acid (VB_9), and 58.4 μg/L inositol. Fermentation was carried out in 100 mL flasks containing 20 mL of corn stover hydrolysate at 42 °C and 150 rpm. The experiments were carried out in duplicate, and the data used here was taken from the average of two parallel experiments.

Shimadzu) with a Bio-Rad Aminex HPX-87H column (Bio-Rad) operated at 65 °C with 0.6 mL/min 5 mM H_2SO_4.

RESULTS AND DISCUSSION

Vitamins B2, B3, and B5: Key Compounds for L-Lactic Acid Production. Vitamin B compounds are essential nutrients for lactic acid bacteria (LAB) to produce lactic acid.[16] To understand the effects of the deficiency of individual vitamin B compounds on the production of L-lactic acid, an engineered strain, *P. acidilactici* ZY271, was used as a model LAB strain, and single vitamin B compounds were eliminated from the synthetic fermentation medium (Figure 1). The synthetic medium used corresponded closely to the commonly used simplified MRS medium containing 10 g/L yeast extract for lactic acid fermentation.[7,16] The deficiency of vitamins B_2, B_3, and B_5 led to approximately 26.2, 20.9, and 33.9% reductions in obtained L-lactic acid titers (40.3, 43.2, and 36.1 g/L), respectively, compared to that with complete vitamin B addition (54.6 g/L). On the other hand, deficiency of vitamins B_1, B_6, B_7, and B_9 and inositol did not lead to observable reductions in final L-lactic acid titers (53.2, 50.5, 51.8, 50.4, and 52.2 g/L). The result indicates that vitamins B_2, B_3, and B_5 are the key vitamins in L-lactic acid production by the lactic acid bacterium *P. acidilactici* ZY271.

This result confirms that vitamins B_2 (riboflavin), B_3 (niacin), and B_5 (pantothenate) are the essential components for cell growth and lactic acid production[16,19−22] VB_5 is incorporated into coenzyme A (CoA) and acyl-carrier-protein (ACP) for protein synthesis and fatty acid synthesis.[30] VB_2 and VB_3 are the precursors of cofactors such as FAD and NAD. NADH levels directly affect lactic acid production as NADH is the cofactor of lactate dehydrogenase.[31]

Elevation of L-Lactic Acid Production by Vitamin B Compounds in Corn Stover Hydrolysate. Lignocellulose biomass contains high amounts of vitamin B compounds,[10] and these biomass derived vitamin B compounds may replace the ones provided by nutrient additives and give substantial substrate cost savings. We conducted L-lactic acid fermentation in corn stover hydrolysate using the LAB strain *P. acidilactici* ZY271 without the addition of vitamin B compounds (see the open black circles in Figure 2); the L-lactic acid titer obtained using corn stover hydrolysate (51.7 g/L after 48 h) was approximately 2.4-fold higher than that obtained using the synthetic medium (21.6 g/L after 48 h, Figure 1) at the same initial sugar concentrations. The result suggests that the vitamin B in the corn stover hydrolysate partly fulfilled the vitamin B requirements of *P. acidilactici* for L-lactic acid production (i.e., it could reduce the needed vitamin supplementation through fermentation nutrient additives such as yeast extract, peptone, or corn steep liquor). When all vitamin B compounds were added into corn stover hydrolysate (see the filled circles in Figure 2), the L-lactic acid titer reached its maximum within 24 h, and the productivity (2.34 g/L·h) was significantly higher than that with complete vitamin B supplementation in synthetic medium (0.76 g/L·h in 72 h), indicating that there were also other unknown beneficial factors in corn stover hydrolysate. Further studies should be focused on exploration of such unknown components in lignocellulose biorefining.

In contrast to the case of L-lactic acid fermentation in synthetic medium, the elimination of vitamin B_2, B_3, and B_5 addition (56.2, 56.5, and 53.7 g/L) did not result in substantial reductions of the final L-lactic acid titer, in comparison with that in the full medium (57.0 g/L, Figure 2). The amount of vitamin B_2 (1420 μg/L) in the corn stover hydrolysate (CSH) was 3 times higher than that in the synthetic medium (495 μg/L, Table 1). Therefore, the amount in CSH could likely satisfy the requirement for the lactic acid fermentation. The vitamin B_3 concentration in CSH (738 μg/L), was 1 order of

Table 1. Vitamin B Content in Corn Stover Hydrolysate

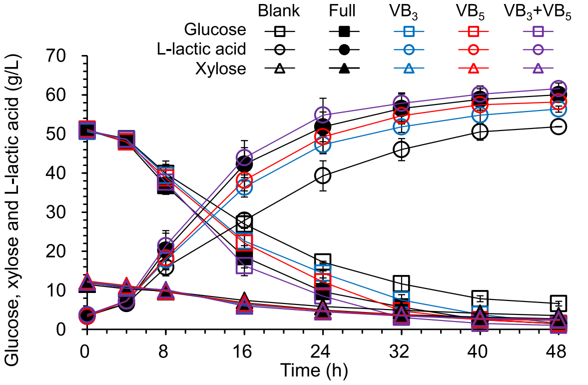
components	control[a] (μg/L)	corn stover hydrolysate (μg/L)
thiamine (VB_1)	954	265
riboflavin (VB_2)	495	1420
niacin (VB_3)	9840	738
pantothenate (VB_5)	2170	30
pyridoxine (VB_6)	772	63
biotin (VB_7)	18	59
folic acid (VB_9)	583	4

[a] The control was medium containing 10 g/L YE, which was assayed by Klotz et al.[16]

magnitude lower than that in the synthetic medium (9840 μg/L), although the L-lactic acid productivity was only slightly lower than that in the full medium (Figure 2). The vitamin B_5 concentration in CSH (30 μg/L) was 2 orders of magnitude lower than that of the synthetic medium (2170 μg/L), which was apparently too low to meet the requirements for efficient lactic acid fermentation. Consequently, vitamin B_5 still needs to be supplemented in cellulosic L-lactic acid fermentations. Because the requirements for the other vitamin B compounds (B_1, B_6, B_7, B_9, and inositol) were found to be not high (Figure 1), the high contents of these vitamins in CSH had no beneficial impact on L-lactic acid production (Table 1). In particular, the content of biotin (vitamin B_7) was substantially higher in CSH (59 vs 18 μg/L).

Promoting Cellulosic L-Lactic Acid Production by Supplementation with Specific Vitamins. We further supplemented the two deficient vitamin B compounds (9102 μg/L VB_3 and 2140 μg/L VB_5) into the corn stover hydrolysate and conducted L-lactic acid fermentation in fermentors with well controlled pH and temperature (Figure 3). After 48 h of fermentation, the L-lactic acid titer was 8.7% higher when vitamin B_3 was supplemented than it was without addition (56.4 vs 51.9 g/L), and the titer was 12.1% higher when vitamin B_5 was supplemented (58.2 vs 51.9 g/L). The values were close to the lactic acid titer in the Full case (i.e., all vitamin B compounds added, 60.0 g/L), and the cell growth rates were similar (Figure 3a,b). The combined supplementation of both vitamins B_3 and B_5 further increased the final L-lactic acid titer to 61.6 g/L. The results suggest that high L-lactic acid fermentation performance in corn stover hydrolysate did not require supplementation with vitamins except vitamins B_3 and B_5, because the other vitamin B compounds as well as micronutrients were present in the feedstock.

(a) L-lactic acid fermentation



(b) Cell viability

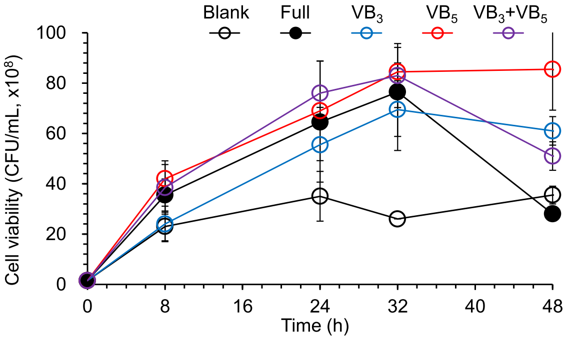


Figure 3. Cellulosic L-lactic acid fermentation using corn stover feedstock with supplementation of specific vitamin B compounds. (a) L-Lactic acid fermentation. (b) Cell viability. Blank, no vitamin B compounds were added; Full, all vitamin B compounds were added according to their contents in synthetic medium; VB_3, only VB_3 was added; VB_5, only VB_5 was added; VB_3+VB_5, both VB_3 and VB_5 were added. Fermentation was carried out in a 3 L fermentor containing 1 L of corn stover hydrolysate. The experiments were carried out in duplicate, and the data used here was taken from the average of two parallel experiments.

Lignocellulose biomass contains high levels of vitamin B compounds, and dry biorefinery technologies such as ammonia fiber expansion (AFEX)[8] and our DryPB technology[6] efficiently preserve considerable amounts of vitamin B compound in the biomass feedstock. The preserved vitamin B compounds could be very important in different cellulosic fermentations. For example, VB_7 (biotin) preserved in the feedstock blocks glutamic acid secretion.[10] This study shows that the vitamin B compounds and micronutrients present in the feedstock can promote L-lactic acid fermentation and also reduce the cost of media by reducing the need for costly exogenous vitamins. Specific supplementation of cellulosic L-lactic acid fermentation with vitamins B_3 and B_5 can further increase the cellulosic L-lactic acid fermentation yield and rate and may therefore replace some of the complex nutrient sources, such as yeast extract and peptone. Chemically synthesized vitamins B_3 and B_5 can be used at the prices of \$9.1 per kilogram of niacin (vitamin B_3) and \$13.9 per kilogram of Ca-pantothenate (vitamin B_5) (≥99% purity, www.1688.com, China). The low dosages (148 g of niacin and 38 g of Ca-pantothenate per ton of L-lactic acid product) only accounted for \$1.9 per ton of L-lactic acid product. More research should be done in the future to preserve more vitamins in lignocellulose feedstock with different pretreatment technologies and further reduce the need to add exogenous vitamins. This study provided a new solution for promoting biorefinery fermentation efficiency and reducing media cost by using vitamin B compounds in lignocellulose biomass.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Detailed procedures of the microbiological assays with the vitamins (PDF)

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Notes

The authors declare no competing financial interest.

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