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Enhance Vitamin B_{12} Production by Online CO_2 Concentration Control Optimization in 120 m^3 Fermentation

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Abstract

Great amounts of carbon dioxide generated by *Pseudomonas denitrificans* during high aerobic vitamin B_{12} fermentation, while the influence of CO_2 concentration on vitamin B_{12} production remains unclear. In this paper, we present parallel experiments to investigate various levels of inlet CO_2 fractions on the physiological metabolism of *P. denitrificans* in laboratory scale fermentation. The results demonstrated that the oxygen transfer rate, cell growth and glucose consumption were inhibited with CO_2 fraction elevated from 0.03% to 8.86 ± 0.24%, while the most exciting results showed that the specific vitamin B_{12} production rate and the yield to glucose were greatly stimulated when dissolved CO_2 increased to 8.86 ± 0.24%. Therefore, the optimal exhausted CO_2 fraction control strategy in 120 m^3 fermenter was established. With the exhaust CO_2 concentration was well controlled at 7.5 ± 0.25% on-line, vitamin B_{12} production greatly improved to 223.7 ± 3.7 mg/L, which was 11.2% higher than that of control. This strategy was proved to be significant necessary and effective for successfully scale up optimization in industrial vitamin B_{12} fermentation.

Keywords: Dissolved carbon dioxide; *Pseudomonas denitrificans;* Vitamin B_{12}; Fermentation; Scale up; Optimization

Introduction

Vitamin B_{12} is an important growth factor and has many applications in medicine and nutrition. Because of its increasing need in the world, vitamin B_{12} has received much more attention recently. Most of the vitamin B_{12} was produced by the culture of aerobic bacterium *P. denitrificans* in industrial production [1]. Due to the actively respiring characters of *P. denitrificans*, the poorly ventilated systems, high back pressure and hydrostatic pressure in large-scale fermenters, high dissolved CO_2 concentration were always encountered in industrial process. As for the significant influence of dissolved CO_2 on the growth and metabolism of many microorganisms [1], it must be taken into consideration in large-scale industrial fermentation.

The effects of CO_2 on growth and product formation in submerged cultures have been investigated in various microorganisms, and the controversy results were always encountered [2,3]. Researches of Gill and Lacoursiere had revealed that cell growth and metabolism of *Pseudomonas fluorescens* and *Escherichia coli* could be stimulated at low dissolved CO_2 level not more than 100 mm Hg pressure or 5% inlet gas phase respectively, while it could be greatly inhibited by the increased CO_2 concentration [4,5]. In yeasts cultivation, the growth rates were seriously decreased under CO_2-enriched condition [6,7]. For filamentous fungi, in which the secondary metabolites formation was always accompanied with morphology changes [1], it was proved that both the morphology and product formation were negatively affected by the elevated CO_2 conditions [1,8,9]. However, many other reports demonstrated that CO_2 could also have positive effect on the metabolism of some microorganisms [10], in which existed high CO_2-fix pathway activity, the anaplerotic reactions were very important for replenishing the metabolites generated from tricarboxylic acid cycle's pool, Researches had shown that enhanced CO_2 could stimulate the activity of key enzymes for the anaplerotic reaction in *Corynebacterium glutamicum* [11,12] and succinate-producing strain [13] by accelerating the accumulation of glutamate and succinate. The former studies on industrial vitamin B12 fermentation have shown that *P. denitrificans* has high affinity to oxygen, and accompanied with higher CO_2 evolution rate during fermentation [14]. However, at present, there was not clearly understood of the effect of CO_2 on metabolism of vitamin B_{12} biosynthesis.

Therefore, the aim of present study was to evaluate the effect of CO_2 on production and metabolism of vitamin B_{12} produced by *P. denitrificans*. Furthermore, an optimal industrial fermentation strategy based on online and CO_2 controlling strategy was successfully applied to enhance industrial vitamin B_{12} production.

Materials and Methods

Microorganisms and medium

The industrial strain *P. denitrificans* used in this study was donated by Huarong pharmacy corporation (Shijiazhuang, China). The cultivations were carried out at a temperature of 32°C and agitation speed of 260 rpm. The seed medium was composed of (g/L): sucrose 40, corn steep liquor 20, betaine 5, $(NH_4)_2SO_4$ 1, $(NH_4)_2HPO_4$ 2, $MnSO_4·H_2O$ 0.8, $COCl_2·6H_2O$ 0.02, MgO 0.3, 6-dimethyl-benzimidazole (DMBI) 0.01, $ZnSO_4·7H_2O$ 0.01, $CaCO_3$ 1.5, the pH was adjusted to 7.2-7.4 by NaOH.

The fermentation medium *lab scale and industrial scale* was composed of (g/L): glucose 80, corn steep liquor 45, betaine 14, $(NH_4)_2SO_4$ 1, KH_2PO_4 0.75, $COCl_2·6H_2O$ 0.075, MgO 0.5, DMBI 0.05, $ZnSO_4·7H_2O$ 0.08, CaCO3 1, pH 7.2-7.4. Feed media (FM) for the fed-batch fermentation were as follows (g/L), FM-1: Glucose 200, DMBI 0.15, $COCl_2·6H_2O$ 0.15 and FM-2: betaine 30, $COCl_2·6H_2O$ 0.3, DMBI 0.3.

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Determination of dry cell weight (DCW) and residual sugar concentration

For determination of biomass concentration, 10 ml ferment broth was centrifuged at 4,000 g for 10 min. After washing twice with distilled water, the cell precipitate was dried to a constant weight at 105°C. The residual sugar concentration was measured by anthrone method [15].

Determination of Pyruvate Carboxylase and intracellular δ-aminolevulinic acid (δ-ALA)

Vitamin B_{12} is a highly complicated molecular structure [16,17] that is biosynthesized from eight molecules of δ-aminolevulinic acid (δ-ALA). This δ- ALA precursor can be generated by either of two pathways (Figure 1): In the C4 pathway, δ-ALA is made from glycine and succinyl CoA by the action of ALA synthase [1]. The δ-ALA was determined according to the literature [18]. For determination of Pyruvate Carboxylase, 10 ml of samples were taken quickly and centrifuged at 4°C, 12,000 g for 5 min, the obtained cells were frozen in -80°C nitrogen. After several times of repeated freezing and thawing, cell pellets were washed with 20 mM Tris–HCl (pH 7.6) for ultrasonication. The sonicated cell suspension was centrifuged and the cell extract was used for the enzyme assay. Pyruvate carboxylase activity was determined by the method of Uy [19].

Measurement of organic acids and amino acids

For the analysis of the extracellular organic acids and amino acids in metabolism, the HPLC systems (Agilent 1100, USA) were equipped with an Aqua Sep C18 (250 × 4.6 mm, 5 μm, ES, USA) and Zorbax Eclipse AAA column (150 × 4.6 mm, 5 μm, Agilent, USA) respectively, the determination conditions were the same to that of previously reported [14].

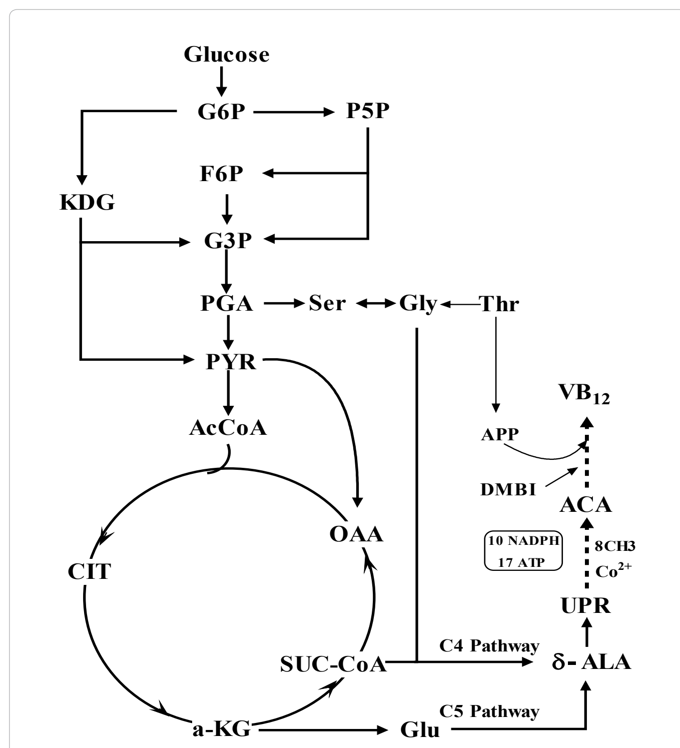


Figure 1: Proposed overview of the pathway of vitamin B12 biosynthesis by *P. denitrificans* connects with central metabolism. Abbreviations: ACA, adenosyl cobyric acid; AcCoA, acetyl-CoA; APP, aminopropanol-o-2-phosphate; CIT, citrate; DMBI, dimethyl benzimidazole; F6P, fructose-6-phosphate; Gly, glycine; Glu, glutamate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; KDG, 2-Keto-3-deoxy-6-phosphogluconate; OAA, oxaloacetate; P5P, pentose-5-phosphate; PGA, 3-phosphate-glycerate; PYR, pyruvate; Ser, serine; SUC-CoA, succinyl-CoA; Thr, threonine; UPR, uroporphyrinogen III; α-KG, α-ketoglutarate; δ-ALA, δ-aminolevulinic acid.

Parameters	
Diameter (T, mm)	3,800
Bottom type	Dished
Filled volume (VL, L)	105,000
Capacity (V, L)	120,000
Baffle length (L, mm)	7600
Baffle width (w, mm)	15
Gas pipe inlet vent diameter (d, mm)	125
Impellers:	
6-Blade concave disc turbine impellers (D, mm)	1,150
3-Blade Rushton disc turbine impellers (D, mm)	1,219
3-Curved-blade disc turbine impellers (D, mm)	1,219
Standard pitched blade disc turbine impellers (D, mm)	1,219

Table 1: Geometric parameters of the 120 m^3 fermenter.

Quantification of vitamin B_{12} in the broth

The vitamin B_{12} concentration was determined by HPLC. Broth samples (25 ml), into which 2.5 ml of 8% $NaNO_2$ and 2.5 ml of glacial acetic acid were added, were boiled for 30 min. Then the upper aqueous phase was measured using HPLC system equipped with C_{18} column (4.6 mm, 25 cm, 5 μm) and UV detector (361 nm). 5% of acetonitrile was used as the mobile phase with flow rate of 1.0 ml min^{-1} at 25°C combined with 95% of 0.25 M sodium acetate anhydrous, the pH was adjusted to 3.6 by acetic acid.

Fermentation process design

Firstly, fed-batch fermentation was carried out in a 50 L turbine-agitated bioreactor (Shanghai Guoqiang Inc., China) with 30 L working volume. The cultivation conditions were same to that of previous report. When the cultivation turned into higher vitamin B_{12} biosynthesis phase, 2.5 L broth was incubated into four parallel 5 L bioreactors respectively (Shanghai Guoqiang Inc., China) for further investigation. Fermentations were performed at 32°C, stirrer speed was 450 rpm, and the pressure in the tar was all kept at 0.1 MP. The inlet gas stream was consisted of a defined mixture of air and pure carbon dioxide with the aeration rate at 1 vvm. The mixture was prepared by thermal mass flow controllers (Mass-Trak, SIERRA, Netherlands). In addition to the control process where air was the input gas, processes were also performed with 3.32 ± 0.12%, 8.86 ± 0.24%, and 13.84 ± 0.27% CO_2 addition (v/v air) in the inlet gas mixture. Meanwhile, the dissolved CO_2 concentration in fermentation was determined with using dissolved carbon dioxide concentration electrode (Mettler).

Fermentation in 120 m^3 fermenter

Three-stage fermentation was performed in 150 L, 9 m^3, and 120 m^3 fermenters gradually, involving two stages of seed growth and one stage of fed-batch vitamin B_{12} fermentation. The primary seed, secondary seed and large-scale fermentation were cultivated with the methods as previous reported [14]. The geometry and dimensions of 120 m^3 fermenter tanks and agitators were given in Table 1.

The inlet and exhaust gas ingredients were analyzed by a mass spectrometer (MAX300-LG, Extrel). Oxygen Uptake Rate (OUR) and Carbon dioxide Evolution Rate (CER) was calculated and collected

online Oxygen Uptake Rate (OUR), and were controlled by adjusting agitation and aeration conditions.

Carbon dioxide control strategy model in 120 m³ fermenter

The culture medium always enriched with CO_2 due to the high evolution rate of growing cells during fermentation process [20], especially in industrial scale fermenters with high hydrostatic pressures, high cell density or high pressure [21,22]. Previous researches revealed that *P. denitrificans* has high affinity to oxygen, with the dissolved oxygen concentration maintained nearly zero, therefore the oxygen transfer rate (OTR) was equal to OUR during the steady fermentation process. The OTR was determined by the agitation, aeration and the pressure, so the equation was used to create their relationship. Meanwhile, the active respiration make the CO_2 levels rise rapidly in large scale fermenters. In order to implement online control the oxygen supply levels and exhausted carbon dioxide concentration, we establish a response model about the OTR and exhausted CO_2 concentration related to aeration and agitation

$$OUR \approx OTR = m \times F^{\alpha} \times R^{\beta} \times P^{r} \quad \text{(Eq.1)}$$

Where m=correction coefficient; α, β, γ=response coefficient of flow, agitation and pressure relate to oxygen transfer rate; OUR= oxygen uptake rate (mmol L/h); OTR=oxygen transfer rate (mmol L/h); F=air flow (L/min); P=pressure (MPa); R=agitator speed (rpm).

Moreover, the relation between oxygen uptake rate and carbon dioxide evolution rate has presented by RQ which shown in Equation 2. In addition, equation 3 shows online determination formula of carbon dioxide evolution rate.

$$RQ = \frac{CER}{OUR} \quad \text{(Eq.2)}$$

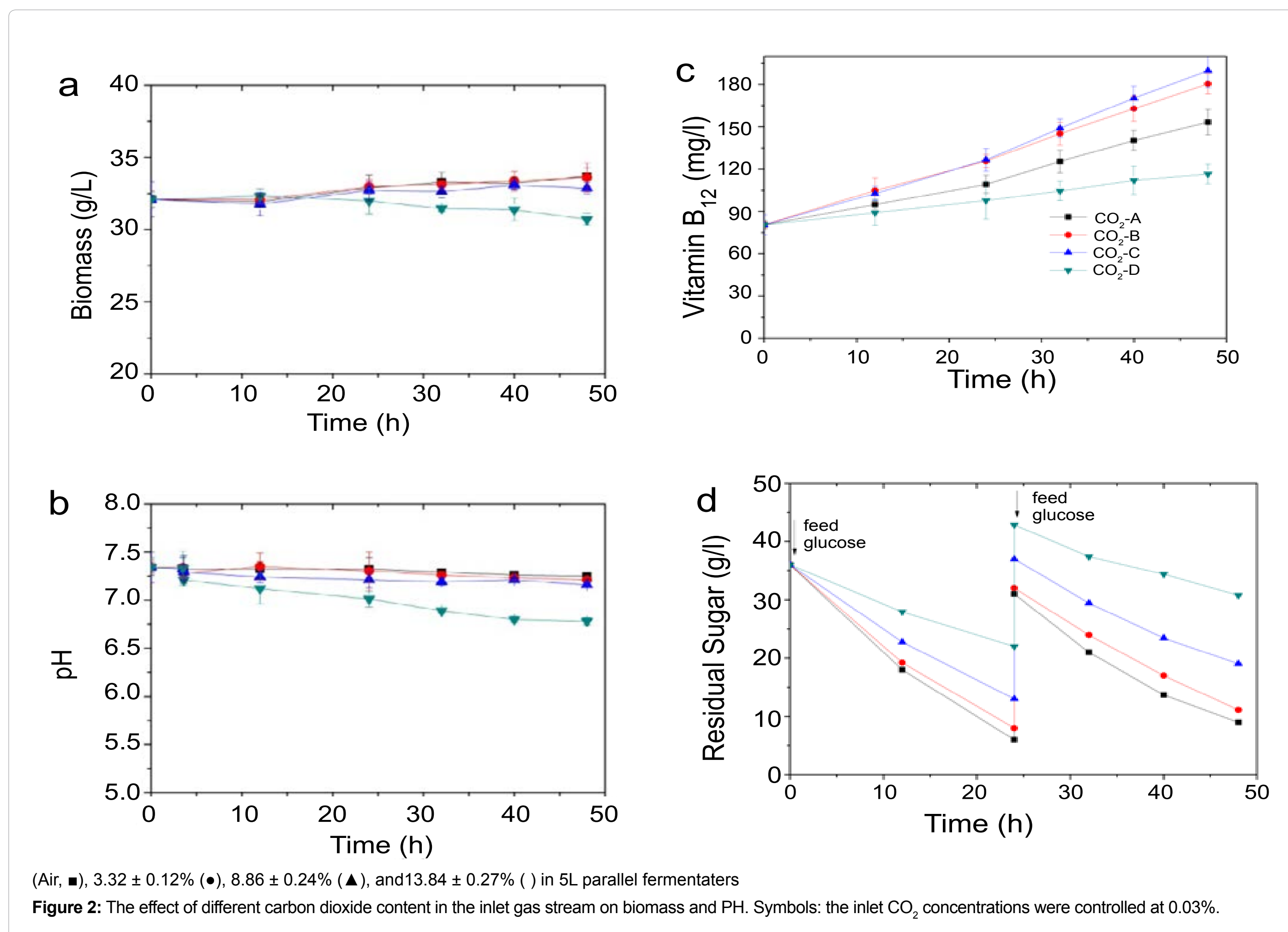
$$CER = \frac{F}{V}\frac{(E_{CO_2} - 0.03)}{22.4} \quad \text{(Eq.3)}$$

Results and Discussion

Effect of CO_2 on the dynamic of vitamin B_{12} fermentation

For investigating the effects of dissolved CO_2 levels on vitamin B_{12} biosynthesis, the stationary cell growth broth in 50 L fermenter was transferred to the four 5 L reactors with 2.5 L working volume simultaneously. The fermentations were performed with operating conditions held constant to ensure that dissolved carbon dioxide was the only major variable. Samples were taken individually for analysis of biomass, glucose, inter-metabolites, and vitamin B_{12} concentrations; and the data points represent mean values from duplicate experiments. A gas-mixing unit was used to give input CO_2 concentration in the influent gas of 0.03%, 3.32 ± 0.12%, 8.86 ± 0.24%, and 13.84 ± 0.27% respectively.

The time course of cell growth and pH under the four different CO_2



(Air, ■), 3.32 ± 0.12% (●), 8.86 ± 0.24% (▲), and13.84 ± 0.27% ( ) in 5L parallel fermentaters

Figure 2: The effect of different carbon dioxide content in the inlet gas stream on biomass and PH. Symbols: the inlet CO_2 concentrations were controlled at 0.03%.

addition were illustrated in Figure 2. During those four conditions, the dissolved oxygen concentration were all maintained at nearly zero levels, while the cell growth haven't any effect when the CO_2 addition elevated from 0.01% to 8.86 ± 0.24%; However, with influent CO_2 concentration increased to 13.84 ± 0.27%, the cell growth rate was greatly inhibited and biomass concentration decreased to 30.7 g/L after 48h cultivation, which was 8.9% lower than that of control (33.68 g/L) (Figure 2a). These might be caused by the respiration inhibition of CO_2 on biological membranes and cytoplasmic enzymes [23], and which may also interact with lipids of the cell membrane, the higher CO_2 concentration would greatly inhibit cell growth [1,9]. It could be clear seen from Figure 2b that pH had hardly any different to the control when CO_2 addition increased to 8.86 ± 0.24%, while the pH was remarkably decreased when the influent CO_2 elevated to 13.84 ± 0.27%.

The effect of influent CO_2 on vitamin B_{12} production rate, glucose consumption rate and specific carbon dioxide evolution rate were summarized in Table 2. Results revealed the inhibitory effects of CO_2 concentration on the specific glucose consumption rate (SGCR). Little decrease of SGCR was appeared with CO_2 addition increased to 3.32 ± 0.12%; However, the SGCR were dramatically decreased to 0.14 and 0.10 mmol/gDCW/h when the influent CO_2 increased to 8.86 ± 0.24% and 13.84 ± 0.27% respectively, which were 19.2% and 46.7% lower than that of control (0.18 mmol/gDCW/h).

Just different from cell growth and SGCR, vitamin B_{12} productions were greatly stimulated when influent CO_2 increased from 0.03% to 8.86 ± 0.24% (Figure 2c). The highest specific vitamin B_{12} production rate reached to 69.4 μg/gDCW/h under 8.86 ± 0.24% CO_2 addition, 53.5% higher than that of control (45.2 μg/gDCW/h). While vitamin B_{12} productions and specific production rate were all significantly inhibited when the CO_2 concentration raised to 13.84 ± 0.27%. Analysis of vitamin B_{12} yields to the glucose consumption ($Y_{p/s}$) (Figure 2d) showed that the maximum yield of 480.5 μg/mmol (vitamin B_{12}/ glucose consumption) were obtained when the influent CO_2 reached 8.86 ± 0.24%, while 13.84 ± 0.27% CO_2 addition led the yields dramatically decreased to lowest levels of 248.3 μg/mmol. Exhaust gas analysis showed that specific

CO_2 fraction	%	0.03	3.32 ± 0.12	8.86 ± 0.24	13.84 ± 0.27
CO_2 conc.	mmol/L	0.001 ± 0.0006	1.16± 0.07	3.05 ± 0.09	4.81 ± 0.12
P_{ra}	ug/L/h	1521.9	2164.2	2279.1	750.0
SPR[b]	ug/ g(DCW)/h	45.2 ± 0.9	64.4 ± 2.1	69.4 ± 1.2	23.7 ± 1.6
SGCR[c]	mmol/ g(DCW)/h	0.18	0.17	0.14	0.10
$Y_{p/s}$	ug/mmol	252.9	382.4	480.5	248.3
SCER[d]	mmol/ g(DCW)/h	1.03	0.90	0.73	0.54
CO_2/Glucose	C(mol)/ C(mol) %	94.6 ± 2.1	89.2 ± 1.9	85.6 ± 1.5	93.3 ± 1.3

[a], *P_r*: Vitamin B_{12} production rate
[b], *SPR:* specific vitamin B_{12} production rate
[c], *SGCR*: specific glucose consumption rate
[d], *SCER:* specific carbon dioxide evolution rate

Table 2: Yield factors of vitamin B12 fermentation under various influent CO_2 concentration for 48h with the cultivations of stationary phase biomass transfered from a well cultured 50L fermentor.

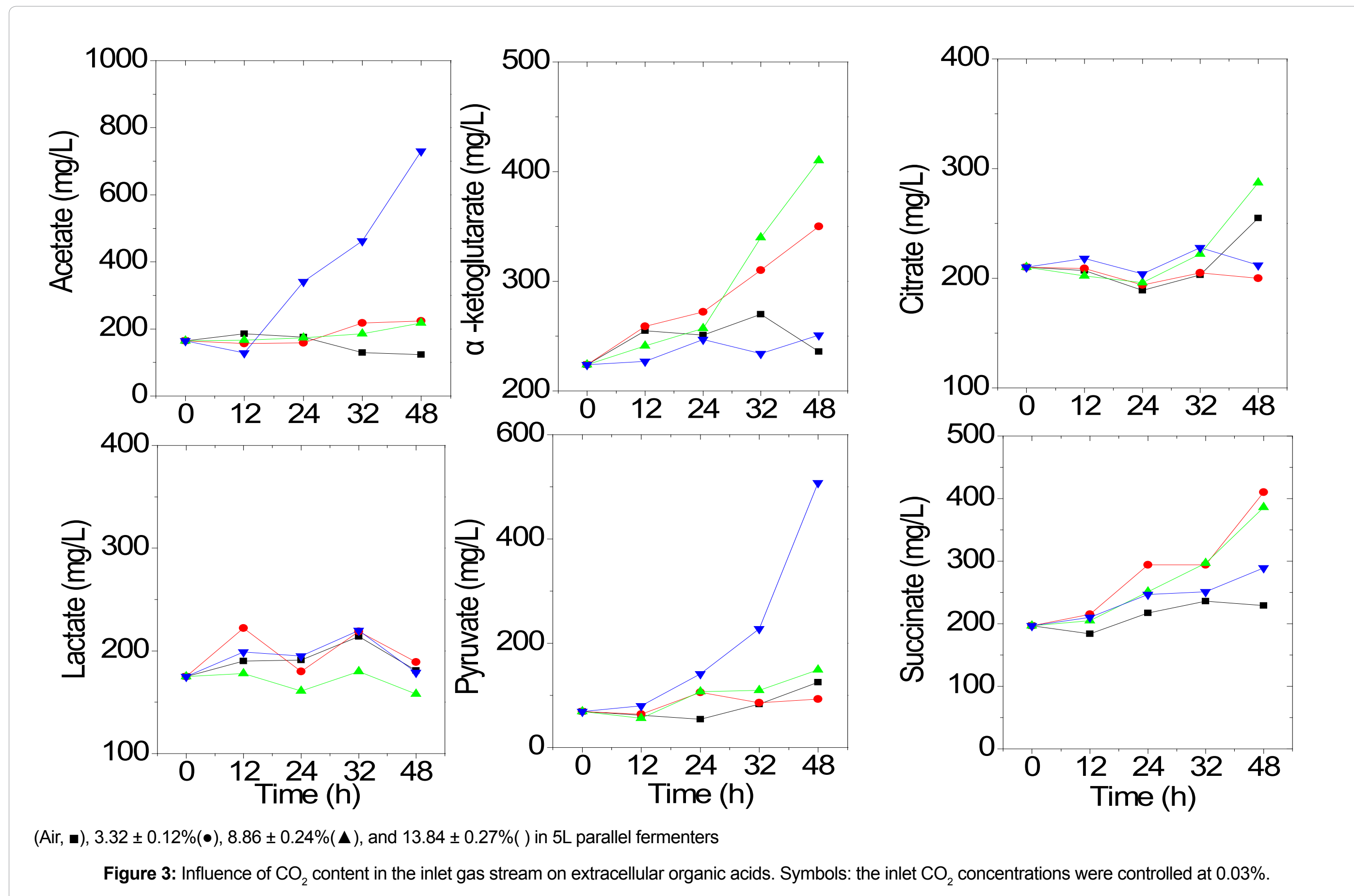


Figure 3: Influence of CO_2 content in the inlet gas stream on extracellular organic acids. Symbols: the inlet CO_2 concentrations were controlled at 0.03%.

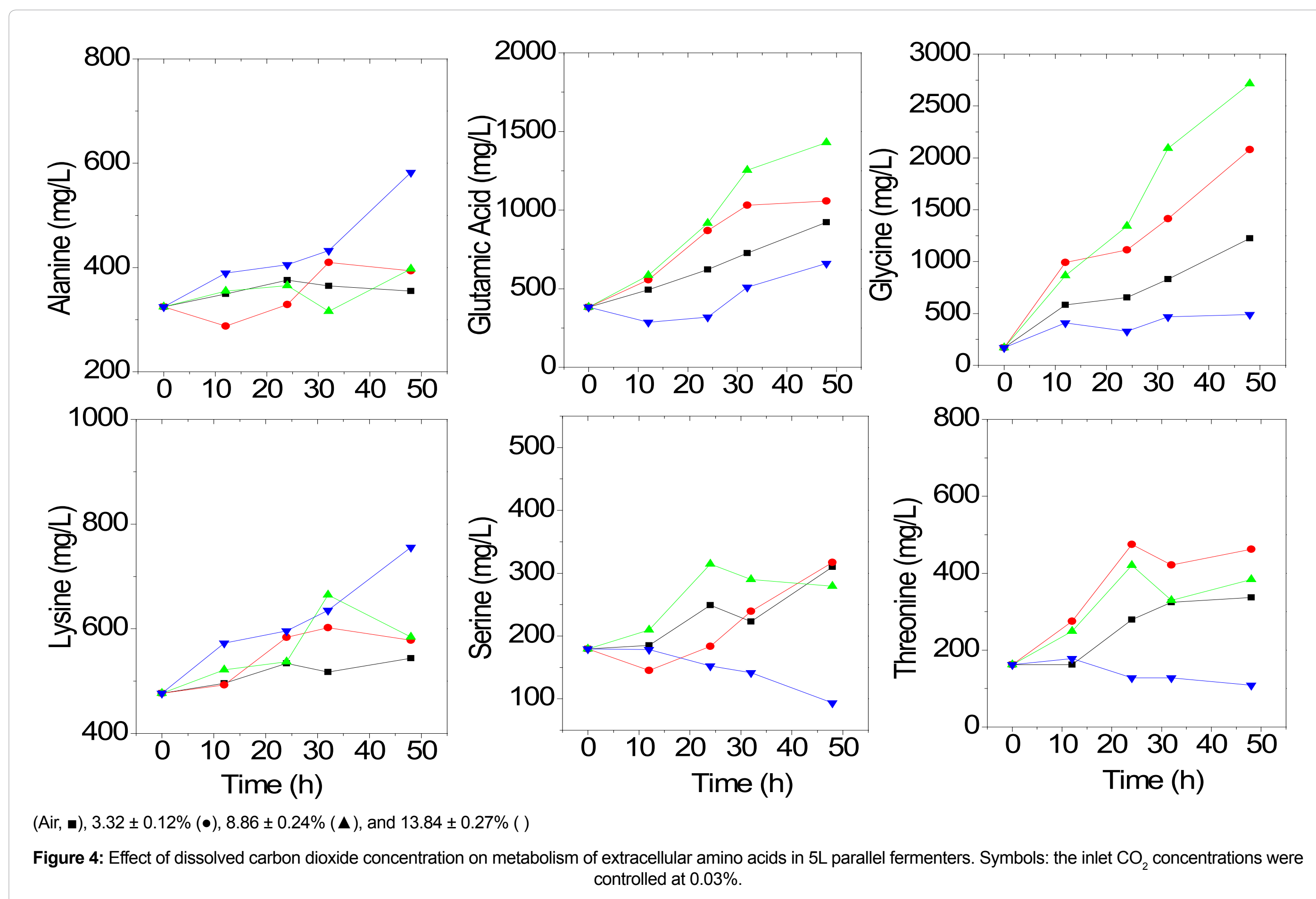


Figure 4: Effect of dissolved carbon dioxide concentration on metabolism of extracellular amino acids in 5L parallel fermenters. Symbols: the inlet CO_2 concentrations were controlled at 0.03%.

carbon dioxide evolution rate (SCER) of *P. denitrificans* decreased with elevated CO_2 concentration in input gas. The statistics of the percent of glucose used for CO_2 generation were decreased from 94 ± 2.1% to 85 ± 1.5% with influent CO_2 increased from 0.03% to 8.86 ± 0.24%; while under the highest CO_2 concentration of 13.84 ± 0.27%, nearly 93.3 ± 1.3% of the glucose consumption was seriously transformed to CO_2. In conclusion, maintained an appropriate input CO_2 concentration around 8.86 ± 0.24% was positive for the economical vitamin B_{12} production.

Previous researches on the effects of elevated CO_2 on the industrial producing strains of bacteria, yeasts, and fungi [1,9] revealed that the inhibitory action of product biosynthesis was probably caused by the decreased substrate consumption with elevated CO_2 concentration. In our research, however, glucose consumption decreased with the increased influent CO_2 concentration, while the yields of vitamin B_{12} based on glucose consumption increased greatly with the elevated influent CO_2 concentration. These results indicated that there must exist abnormal metabolism characters of *P. denitrificans* under the various influent CO_2 concentrations. The reasons were further investigated by organic acid and amino acid analysis of the central metabolism.

Effect of CO_2 on the inter-metabolites in fermentation

The researches on vitamin B_{12} biosynthesis had demonstrated that the concentrations of the precursor of δ-aminolevulinic acid (δ-ALA) played an important role on vitamin B_{12} biosynthesis [16]. δ-ALA could be synthesized from C4-pathway (from glycine and succinyl CoA by δ-ALA synthesis) or C5-pathway (from glutamate by glutamyl-tRNA reductase) associated with central metabolism [24]. The dynamic processes of extracellular organic acids concentration under different CO_2 levels were shown in Figures 3 and 4.

When influent CO_2 increased from 0.03% to 8.86 ± 0.24%, α-ketoglutarate and succinate concentrations greatly increased, which were actually accelerated the biosynthesis of glutamate, and the other organic acids such as pyruvate, acetate, lactate and citrate hadn't any different. However, the acetate and pyruvate were accumulated much higher when influent CO_2 reached 13.84 ± 0.27%. These were probably caused by the inhibition of respiration under high CO_2 concentration, and the resulted high NADH accumulation in the cell really repressed the activity of pyruvate dehydrogenase and TCA cycle, and then attributed to the accumulation of acetate and pyruvate.

The dynamic analysis on the concentrations of extracellular amino acids (Figure 3) showed that glutamate and glycine synthesis rate was significantly enhanced with elevated CO_2 concentration. Under 8.86 ± 0.24% CO_2 addition, glutamate and glycine concentration reached 1430.2 mgL^{-1} and 2716.4 mg/L, respectively, which were 55% and 122% higher than that of control. Threonine reached highest synthesis rate when inlet CO_2 increased to 3.32 ± 0.12%. These metabolites accumulation could be accelerate the precursor's biosynthesis for vitamin B_{12} synthesis.

δ-ALA is the critical precursors of vitamin B_{12} biosynthesis, the time course of δ-ALA concentration under different levels of inlet CO_2

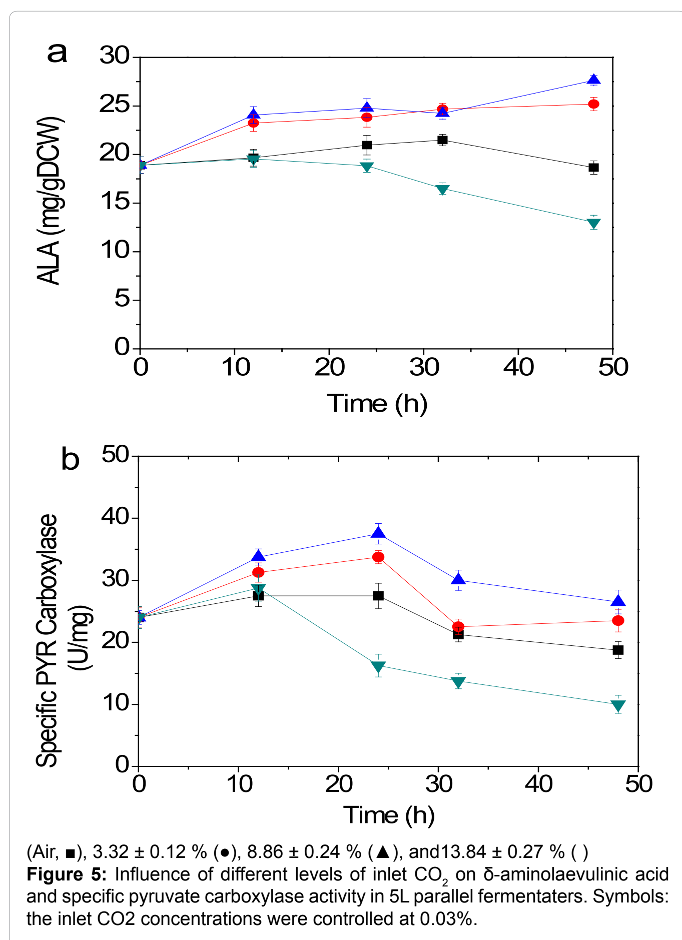
were shown in Figure 5a, experiment results demonstrated the positive effects of moderate CO_2 concentration exerted to the δ-ALA synthesis. In bioprocess sparged with 8.86 ± 0.24% CO_2 addition, the δ-ALA concentration reached the top point at 26.4 ± 1.2 mg/gDCW, which was 40.4% higher compared to that of control (18.6 ± 0.9 mg/gDCW). However, the δ-ALA biosynthesis was seriously inhibited with13.84 ± 0.27% CO_2 addition, the final δ-ALA concentration was lowest as 13.02 ± 0.5 mg/gDCW, 30% lower than that of control.

Experiment results showed that the biosynthesis of glycine, succinic acid, and glutamate were elevated with the increased influent CO_2 concentration from 0.03% to 8.86 ± 0.24%, which actually augmented δ-ALA generation for higher vitamin B_{12} biosynthesis.

Effect of CO_2 on pyruvate carboxylase activity

Previous researches have illustrated that anaplerotic reaction catalysed by pyruvate carboxylase was mostly existed for replenishing the TCA cycle in *Pseudomonas* spp [25]. In this work, the dynamic analyses of specific pyruvate carboxylase activity in different input CO_2 addition were shown in Figure 5b. In the process sparged with 8.86 ± 0.24% CO_2, the maximal specific activity reached 37.2 U/gDCW, which was 35% higher than that of control (27.5 U/gDCW).

Tricarboxylic acid (TCA) cycle is one of the main central pathways in aerobic bacteria. It is responsible for the complete oxidation of acetyl-CoA and provision of precursors for many TCA-related metabolites biosynthesis. As precursors of the metabolites succinyl-CoA and glutamate for δ-ALA biosynthesis were all generated from



(Air, ■), 3.32 ± 0.12 % (●), 8.86 ± 0.24 % (▲), and13.84 ± 0.27 % ( )

Figure 5: Influence of different levels of inlet CO_2 on δ-aminolaevulinic acid and specific pyruvate carboxylase activity in 5L parallel fermentaters. Symbols: the inlet CO2 concentrations were controlled at 0.03%.

F(L/min)	R(rpm)	P(MPa)	OUR (mmol/L/h) Determined	Predicteda
1263.1	78	0.031	47.38	45.71
929.9	78	0.034	40.66	41.77
406.4	50	0.031	24.73	24.20
600.5	90	0.041	39.10	39.72
850.4	78	0.032	37.51	39.80
1523.2	110	0.040	63.2	61.68
1224.5	81	0.032	46.07	46.41
746.3	79	0.029	36.65	37.23
960.0	55	0.035	38.42	36.21
959.4	78	0.033	40.77	41.95
709.2	77	0.028	36.74	35.77
625.5	81	0.033	42.58	36.39
1121.8	78	0.027	43.82	42.32

a calculated from the obtained equation 2 with the corresponding process parameters

Table 3: The determined and predicted OUR values at various aeration, agitation and pressure levels in the 120 m^3 bioreactor.

TCA cycle. In the rapid VB_{12} synthesis phase, the relative high demand of succinyl-CoA and glutamate lead to an "overflow" of corresponding compounds of TCA cycle. Therefore, TCA cycle has to be replenished continuously through anaplerotic reaction for oxalacetate generation to maintain the cycle running.

The experiment results demonstrated that elevated levels of CO_2 concentration had significant effect on vitamin B_{12} fermentation. When the influent CO_2 concentration increased from 0.03% to 8.86 ± 0.24%, the specific vitamin B_{12} production rate and the yields of vitamin B_{12} to glucose were stimulated. However, cell growth, glucose utilization, specific vitamin B_{12} production rate, and the yields of vitamin B_{12} to glucose were all greatly inhibited when CO_2 addition up to 13.84 ± 0.27%. Therefore, the influences of CO_2 on industrial vitamin B_{12} production have to take into consideration.

Industrial fermentation strategy based on online exhaust CO_2 controlling

Exhaust CO_2 concentration controlling strategy: With the process parameters determined under various agitation speed, air flow, and pressure obtained from 120 m^3 fermentation tank are shows in Table 3, we obtained relevant respond coefficient through regression analysis of these parameters between OTR and Process parameters determined (Eq.1). Then, oxygen transfer rate and oxygen uptake rate could be calculated according to these parameters (Eq.4).

$$OUR \approx OTR = 1.0074 \times F^{0.3729} \times R^{0.4635} \times P^{0.2472} \quad \text{(Eq.4)}$$

The carbon dioxide fraction in exhausted gas was calculated from a mutual relation between RQ, CER, and OUR (Eq. 2,3,5)

$$Eco_2 = RQ \times F^{-0.36271} \times R^{0.4635} \times P^{0.2472} \times V \times 22.57 + 0.03 \quad \text{(Eq.5)}$$

During the vitamin B_{12} fermentation process, glucose was used as the main carbon substrate, the respiration quotient (RQ) of this strain was always maintained at 1.03 ± 0.02 in microorganism growth period and RQ was calculated as 0.95 ± 0.04 in vitamin B_{12} synthesize period. Then OUR and exhausted CO_2 concentration from 120 m^3 fermentation tank could be obtained by adjusting agitator speed and air flow according to the created model above (Figure 6).

Scale-up fermentation based on exhaust CO_2 concentration control: Based on the results obtained from 5 L vitamin B_{12} fermentation, the optimal controlling strategies based on exhausted

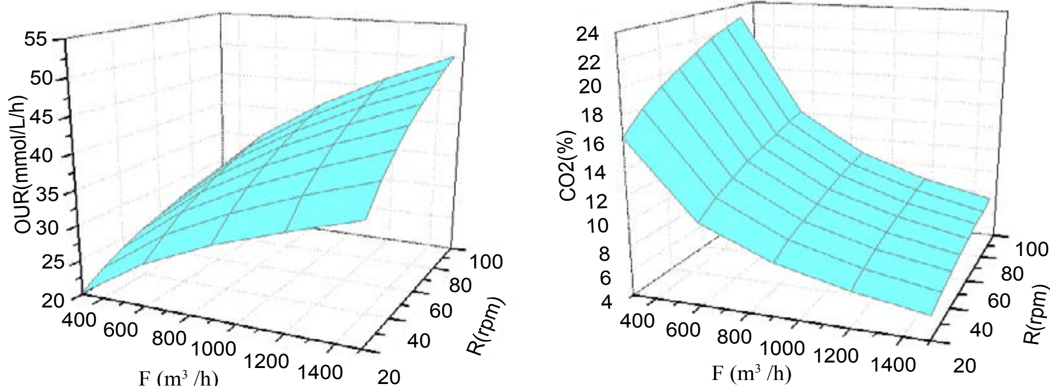


Figure 6: The response of oxygen uptake rates and exhaust CO_2 concentrations as a function of aeration and agitation in120 m³ large scale fermenter (based on the obtained equation4 and equation 5.

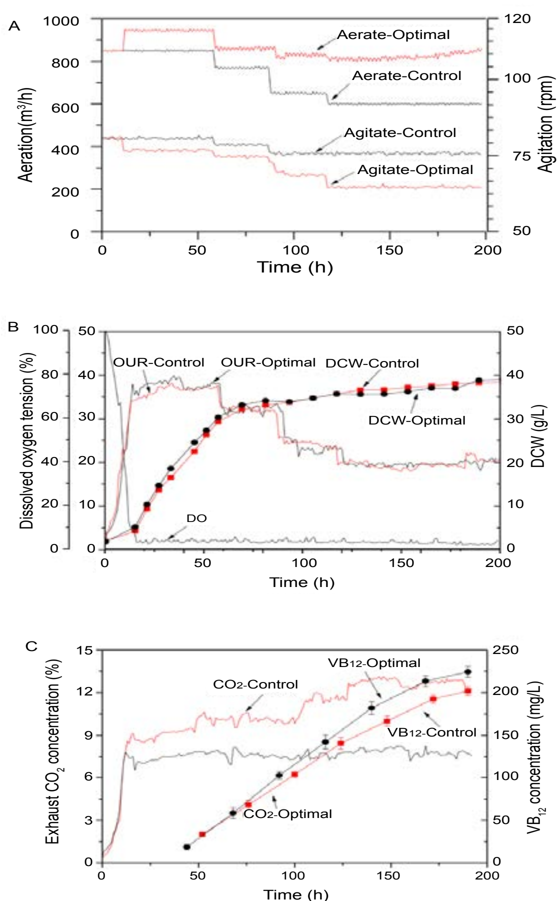


Figure 7: Time course of industrial VB12 fermentation with the exhaust CO_2 concentration controlled at throughout at 7.5 ± 0.25 %, (optimal) and the control without exhaust CO_2 controlled (control) in 120 m³ fermenters and the effect of CO_2 concentration on DCW (b) and VB_{12} concentration (c) by correlation adjustment of oxygen supply conditions (a).

CO_2 concentration were implemented for vitamin B_{12} fermentation in 120 m³ fermenters. The exhausted CO_2 concentration was controlled at 7.5 ± 0.25% by adjusting aeration and agitator speed, also OUR was maintained at needed level. This strategy greatly prevented the inhibition of high dissolved CO_2 concentration on vitamin B_{12} synthesis.

Time course of OUR, dry cell weight, vitamin B_{12} production, and exhausted CO_2 concentration were shown in Figure 7. By control the aeration and agitation throughout the fermentation process, the changes of OUR were maintained at similar levels in fermentation processes (Figure 7b), the optimal strategy by stepwise reduction of oxygen uptake rate under dissolved oxygen limiting level during fermentation process was used for oxygen supply levels control [11]. Meanwhile, the exhausted CO_2 concentration changed greatly and maintained at the suitable levels with the adjustment of agitation and aeration conditions. In the contrast conditions, the exhaust CO_2 concentration increased greatly to 9.1 ± 0.15%, cell growth rate was lower than that of optimal strategy, especially when it elevated to 12.0 ± 0.24% after 75 h cultivation, the vitamin B_{12} production rate was gravely inhibited (Figure 7c).

The results presented demonstrated that cell growth and vitamin B_{12} production were greatly stimulated by controlling a lower CO_2 concentration of 6.0 ± 0.24% during the early fermentation phase. The highest vitamin B_{12} production rate was achieved when exhausted CO_2 concentration were controlled at 7.5 ± 0.25%, The maximum vitamin B_{12} production reached 223.7 ± 3.7 mg/L at 190 h, which was 11.2% higher than that of without CO_2 concentration controlled (201.4 ± 2.9 mg/L). It can be observed that higher production would be realized through simultaneously regulate the appropriate OUR and CO_2 concentration levels are more beneficial to vitamin B_{12} synthesis.

Conclusion

CO_2 was produced in aerobic and anaerobic fermentations through decarboxylation reactions in a number of metabolic pathways, and hence has an influence on the performance of microbial cultivations [1,9]. Especially in industrial large bioreactors with high total pressure, poorly ventilated system, and inefficiently aerated fermenters. In this work, we investigated the effects of dissolved CO_2 concentration on vitamin B_{12} fermentation of *P. denitrificans,* established the correlation of exhausted CO_2 concentration and the activity of the microbial metabolism by inter-scale observation and data association. The novel and optimal fermentation controlling strategy based on OUR and exhausted CO_2 concentration was successfully applied in industrial scale up of vitamin B_{12} fermentation.

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