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Full Length Research Paper

An optimum medium designed and verified for alcohol vinegar fermentation

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In this article, a novel formula of nutrient salt for alcohol vinegar production was derived based on mass conservation theory of carbon source, nitrogen source and inorganic ions. Series of semi-continuous fermentations were successfully carried out in a Frings 10 L fermentation tank. The average acetification rate of semi-continuous fermentation was 2 g acetic acid/L*h within about 12 h, average stoichiometric yield was 94%, and then fed-batch trial for high concentration vinegar fermentation was successfully conducted. Both of these confirmed that this method applied was cost-effective. If this method is adopted in industry scale, vinegar manufacturers will reduce production cost significantly.

Key words: Formula, alcohol vinegar, fermentation nutrient.

INTRODUCTION

Vinegar, as a wine by-product, has lately acquired an important role in salad dressings, ketchup and other sauces, etc (De Ory et al., 1999; Bartowsky et al., 2008). It is industrially produced largely by semi-continuous process where a fraction of the total volume of submerged culture is withdrawn on a periodic basis. The remaining volume acting as an inoculum for the next cycle is replenished with fresh wine added in proper portions to obtain the final working volume. This procedure causes temporal changes in the properties of the culture medium where acetic acid bacteria grow (De Ory et al., 2004).

Acetobacter pasteurianus is a Gram-negative, aerobic, rod-shaped acidophilic bacterium, which is known for its incomplete oxidation of a wide range of carbohydrates and alcohols at low pH by its membrane-bound dehydrogenases (Buchanan and Gibbons, 1974; Cleenwerck and De Vos, 2008). The corresponding oxidative products are completely secreted into the medium making it suitable for industrial application. However, there is a bottleneck problem: the optimized quantity of nutrient salt used by acetobacter for alcohol vinegar production. Berraud (2000) researched the quantity of nutrient salt utilized when fermenting the highly concentrated alcohol vinegar. The total was 0.3% g nutrient per liter. Frings Corporation reduced the total quantity to 0.2% (Frings Company, 2004). Because this nutrient salt development is based on *Gluconacetobacter* (Dellaglio et al., 2005), there is no specific wine vinegar nutrient salt for *A. pasteurianus*. The vinegar production amount of China is 2 million tons every year, as the largest vinegar making country of the world, hence China has a big potential nutrient salt market.

Carbon source, nitrogen source, inorganic ions and vitamins are significant living factors for bacteria (Camu et al., 2007). Glucose, YEP (yeast extract powder), KH_2PO_4, $MgSO_4$, and NaH_2PO_4 have been chosen as the nutrient salt conponents by vinegar fermentation experiments with flask. In order to make nutrient salt suitable for industrial process, the quantity must be optimized. In this work, a novel fast formula for estimating economic quantity of nutrient salt was developed according to mass conservation. Then semi-continuous and fed-batch

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fermentation trials were conducted to confirm this formula separately, so that optimum amount of nutrient salt could be fast figured to reduce cost for industrial applications.

MATERIALS AND METHODS

Microorganisms

A. pasteurianus HN101 was utilized as seed which was isolated from a fully operational industrial fermentation tank. The total dry cell concentration during a cycle ranged from 0.36 to 0.6 g/L.

Substrate

The main media were 95% (v/v) ethanol, production water and nutrient salt. The quantity of nutrient salt before optimizing were glucose (5 g/L), YEP (3 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4$ (0.5 g/L) and NaH_2PO_4 (0.5 g/L), which were well mixed before filling into tank.

Fermentation instrument

The experiments were implemented in a Frings 10 L fermentation tank modeling industrial vinegar fermentation. The bioreactor was operated in an automated manner (loaded, unloaded, controlled and monitored without operator intervention via previously programmed computer software).This procedure resulted in temporal changes in some operational variables including volume, ethanol concentration, acidity and cell concentration.

Batch fermentation

Inoculation was developed with 0.5 L of selected medium culture until the exponential growth phase. This inoculum was mixed with 4.5 L of substrate [ethanol (40 g/L), acetic acid less than 5 g/L and nutrient salt]. The amount of nutrient salt was excessive. Constant temperature was set at 30°C. The passing air was at a flow-rate of 0.1 vvm (per liter medium per hour). The whole process ended with no acidity change.

Semi-continuous fermentation

0.3 L of the medium selected at the exponential growth phase was taken out from the shake flask as inoculum mixed with 2.7 L of the substrate (25 g/L ethanol with quantified nutrient salt). When acetic acid concentration reached 20 g/L, 3 L of the substrate (50 g/L ethanol and quantified nutrient salt) were added to the fermentation. Then, when the acetic acid got to 45 g/L, the last 2 L of the substrate (70 g/L ethanol and quantified nutrient salt) were mixed (De Ory et al., 2002). The total time was 2 days and the total volume was 8 L containing acetic acid (60 g/L). The bacteria was kept growing to the exponential phase with a high fermentative potential during this procedure. The passing air was at a flow-rate of 0.1 vvm.

Cyclic vinegar fermentations were developed in the Frings 10 L fermentation tank. When acetic acid concentration reached about 60 g/L, 2.4 L of the fermented mash were discharged and refilled with the same volume of fresh mash (50 g/L ethanol and quantified nutrient salt).The resulting medium contained about 40 g/L acetic acid and 25 g/L alcohol for the next cycle (Drysdale and Fleet, 1985; Bar et al., 1987). The passing air was at a flow-rate of 0.08 vvm.

Fed-batch fermentation for high concentration

High concentration vinegar fermentation was developed by fed-batch style in the Frings 10 L tank. The initial volume of mash was 6 L which contained acetic acid (22 g/L), alcohol (32 g/L) and nutrient salt. The medium selected at exponential growth phase (10% of starting mash volume) was inoculated into the reactor. When the residual alcohol concentration in tank was reduced less than 6 g/L, some substrates (alcohol and nutrient salt) were fed. The operations are presented in Table 6.

Analysis methods

Ethanol by ALKOSENS which could detect the concentration of ethanol on line; acetic acid (Fregapane et al., 2001) by titration with a 0.1 M NaOH solution, and phenolphtaleine indicator, organic acids different to acetic acid was considered non-relevant for titration. Glucose was detected (Wen et al., 2005) by a glucose biosensor SBA-40C (Biological Institute of Shandong Academy of Sciences, China) and α-amino nitrogen (Botella et al., 1990) by a HPLC instrument.

Biomass measurement

Total biomass (Wei et al., 2009) (cell growth) was monitored by optical density measurements at 600 nm using a UV/visible spectrophotometer. Samples were diluted to the appropriate concentration to keep the OD_{600} nm value between 0.3 and 0.8. ($1OD_{600}$ = 0.45 g dry cell weight/L)

RESULTS AND DISCUSSION

Batch fermentation experiment

For the purpose of getting the relationship between bacteria growth and substrate depletion, batch fermentation of vinegar with *A. pasteurianus* was carried out.

Figure 1 shows that the batch process had four phases according to the growth curve. Phase I (0 to 12 h) was the lag phase; cells utilized substrate to synthesize co-metabolic enzymes and provided maintenance consumption without obvious biomass increase, Phase II (12 to 24 h) was the logarithmic phase; maximum specific growth rate reached 0.32 h and acetification rate gradually accelerated, Phase III (24 to 32 h) was the deceleration phase; the ratio of acid production rate was still at a high level (8.5 g acid/g cell*h) when specific growth rate decreased and Phase IV (32 to 36 h) was the stable phase; biomass was kept constant and acetic acid concentration was not changed until alcohol was exhausted in the end.

Figure 2 depicts the variations of glucose and α-amino nitrogen during the batch fermentation which was well connected to the growth curve (Figure 1). When fermentation arrived at Phase IV, α-amino nitrogen was not decreased apparently, but glucose was still consumed to provide and maintain energy for living. So, substrate consumption of bacteria metablism was calculated by cells yield coefficient (C_{YC}).

Table 1. C_{YC} to different substrates.

C_{YC}	$Y_{X/C}$	$Y_{X/N}$
Equation	$\frac{X_t - X_0}{C_0 - C_t}$	$\frac{X_t - X_0}{N_0 - N_t}$

Table 2. Main inorganic components of bacteria.

Element	Maximum level (g/100 g dry biomass)
P	3.0
S	1.0
K	4.5
Mg	0.5
Na	1.0
Ca	1.1

The general formula of C_{YC} is the equation:

$$Y_{X/S} = \triangle X/\triangle S \quad (1)$$

According to the batch fermentation data and Equation (1), $Y_{X/C}$:0.2 (g biomass/g glucose) and $Y_{X/N}$:8.3 (g biomass/g α-amino nitrogen) was calculated (Table 1).

$Y_{X/S}$ is the cells yield coefficient to substrate, ΔX (g/L) is the changed biomass between a given period of fermentation glucose, Δ*S* (g/L) is the consumed substrate versus ΔX.

So, based on the batch fermentation data, the $Y_{X/C}$ and $Y_{X/N}$ were calculated as follow:

$$Y_{X/C} = \frac{X_t - X_0}{C_0 - C_t} = 0.21$$

$$Y_{X/N} = \frac{X_t - X_0}{N_0 - N_t} = 8.25$$

Where, X_t (g/L) is the biomass at a given time (t=32 h) in the experiment process, X_0 (g/L) present the biomass at the start, similarly C_0 (g/L) was the glucose concentration before inoculation, C_t (g/L) was the glucose concentration versus X_t, N_0 (g/L) was the initial α-amino nitrogen concentration and N_t (g/L) was the α-amino nitrogen concentration when fermented 32 h.

Besides, organic components bacteria also require inorganic elements including sulfur, potassium, magnesium, sodium and calcium etc. As relative literature, Lee et al. (2003) reported the maximum levels (Table 2).

In the industrial level of submerged fermentation, about 1% (v/v) alcohol could be transformed into 1% (m/v) acetic acid (De Ol et al., 2002). Based on tolerated acid concentration of bacteria, Y_A (yields of acetic acid to biomass) was 120 g acetic acid/g dry cell; the quantity of nutrient salts for producing a certain concentration of acetic acid was inferred, the details are as follow: if the concentration of vinegar is M (g/L), the dry biomass of cells (g/L) required will be:

$$m = M / Y_A \quad (2)$$

According to Equation (2) the mash dosages (g/L) of various nutrients needed were calculated by mass conservation theory before fermentation. The results are shown in Table 3.

The optimal total dosage (g/L) of each nutrient added was concluded and the formula is given as follows:

$$\frac{M}{Y_A}\left(\frac{1}{Y_{X/C}}(glu\cos e) + \frac{20.8}{Y_{X/N}}(YEP) + 0.037(MgSO_4) + 0.053(NaH_2PO_4) + 0.157(KH_2PO_4)\right)$$

Semi-continuous experiments

Starting-up of the vinegar fermentation is a key aspect for the whole production. Appropriate operation of this process is necessary (De Ory et al., 2002).

The starting-up procedure is shown in Figure 3. When acetic acid bacteria grew to the exponential phase, with high acid productivity and significant decreased ethanol concentration, some fresh medium (alcohol and some nutrient salt) was added. The quantity of nutrient salt was determined by the formula above; the data are presented in Table 4. The microorganism grew optimally without appreciable lag phase during the starting-up process. This method decreased the time to 48 h, so vinegar fermentation could start in a short time.

Some cycles of fermentation were developed after the starting-up. When the final concentration of acetic acid was 65, 2.4 L of vinegar were discharged and replaced with 2.4 L of wine. Figure 4 show the total acidity and ethanol concentration versus time for the three fermentation cycles and the quantity of nutrient salt added every cycle was presented (Table 5).

As could be observed, fermentation cycles developed smoothly with quantitative nutrient salt was figured by the deduced formula in a short time. The average acetification rate for the fermentation cycles was 2 g acetic acid/L*h with 12 h as average cycle duration. Maximum value of the acetification rate in a fermentation cycle was about 3.5 g acetic acid/L*h. Mean stoichiometric yield was 94%, thanks to the automatic control system which discharged the vinegar in time.

Fed-batch experiment for high concentration As shown in Figure 5, high concentration of vinegar

Table 3. Nutrient salt requirement for quantified concentration of vinegar.

Nutrient	m	$m_{Glucose}$	m_{YEP}[a]	m_{MgSO4}[b]	$m_{NaH2PO4}$[c]	m_{KH2PO4}[d]
Equation	$\frac{m}{Y_{X/C}}$	$\frac{m}{Y_{X/C}}$	$\frac{m}{Y_{X/N}\times 0.048}$	$\frac{m\times 0.01}{0.267}$	$\frac{m\times 0.01}{0.19}$	$\frac{m\times 0.045}{0.287}$

[a],α-amino nitrogen content of YEP was 4.8% in this work. Because the production water contained lots of magnesium and calcium which met bacteria requirment, $MgSO_4$, NaH_2PO_4,KH_2PO_4 were only chosen to be added; [b],the weight of sulfur element in $MgSO_4$ was 26.7% (m/v); [c], sodium element in NaH_2PO_4 was 19%; [d], potassium element in KH_2PO_4 was 28.7%.

Table 4. Quantity of nutrient salt added during the start-up.

Substrate (g/L)	Glucose	YEP	$MgSO_4$	NaH_2PO_4	KH_2PO_4
Step 1	1.25	0.6	0.01	0.013	0.039
Step 2	1.4	0.7	0.01	0.015	0.04
Step 3	1.13	0.56	0.008	0.012	0.035

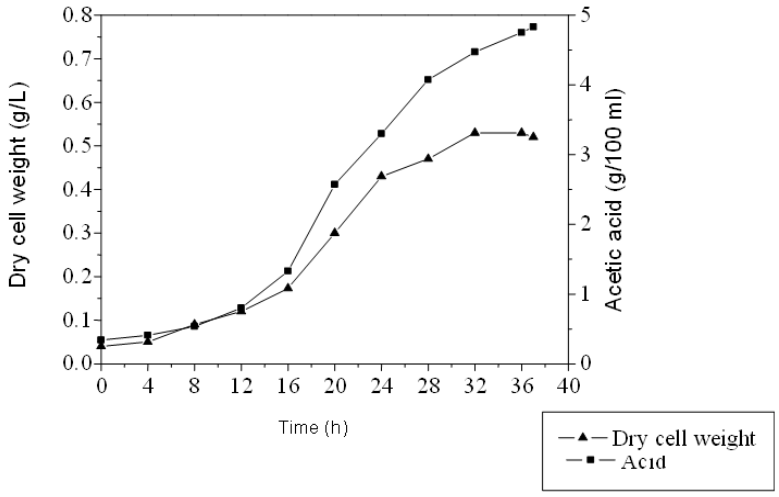

Figure 1. Batch fermentation experiment.

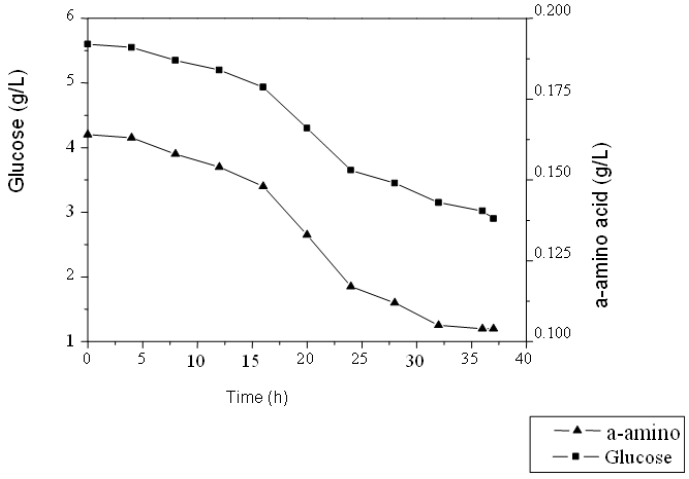

Figure 2. Process of nutrient consumption.

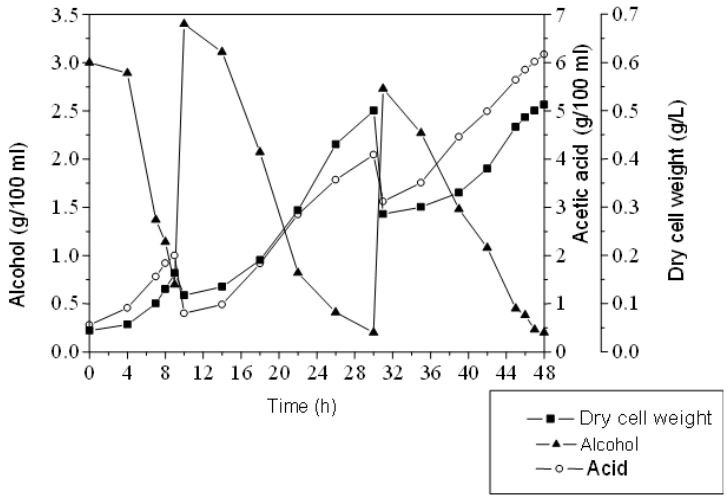

Figure 3. Starting-up protocol.

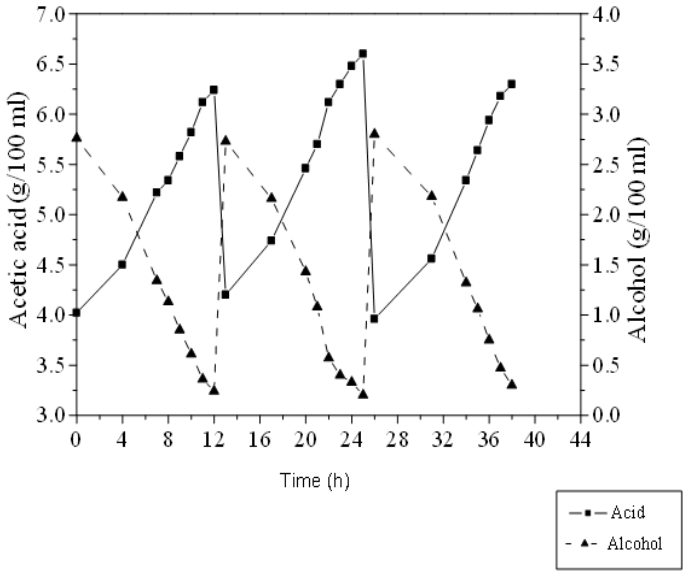

Figure 4. Semi-continuous fermentation experiments.

Table 5. Quantity of nutrient salt added of each cycle.

Substrate(g/L)	Glucose	YEP	$MgSO_4$	NaH_2PO_4	KH_2PO_4
Cycle 1	1	0.53	0.008	0.01	0.037
Cycle 2	1.1	0.55	0.008	0.012	0.035
Cycle 3	1.17	0.58	0.009	0.012	0.036

was made by the fed-batch fermentation; the final acetic acid concentration was 116 g/L, there was no lag phase after the addition of the substrate every time which explained that the formula was appropriate for alcohol vinegar production. The blank bar represents the alcohol concentration after every fed-batch operation.

Conclusions

A. pasteurianus HN101 is an energetic strain for industrial vinegar fermentation. The yield of acetic acid to biomass was higher than for some other strains; so optimizing the added nutrient salt is necessary for

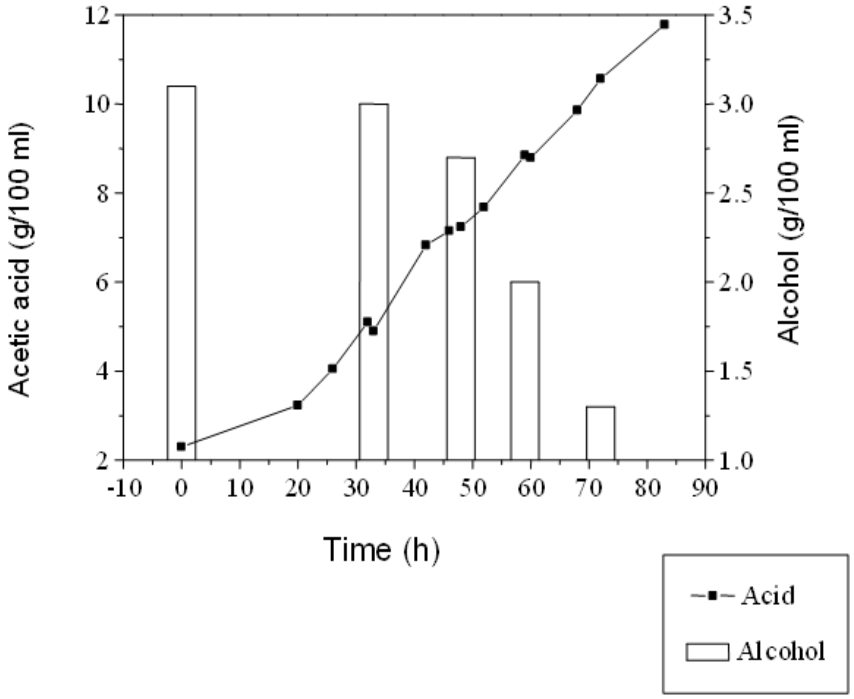

Figure 5. Fed-batch fermentation experiment.

Table 6. Quantity of nutrient salt added during the fed-batch fermentation.

Substrate(g/L)	Alcohol	Glucose	YEP	$MgSO_4$	NaH_2PO_4	KH_2PO_4
Initial	32	1.33	0.68	0.01	0.014	0.04
Fed 1	30	1.25	0.63	0.01	0.013	0.039
Fed 2	27	1.13	0.57	0.008	0.012	0.035
Fed 3	21	0.88	0.44	0.0065	0.009	0.027
Fed 4	13	0.54	0.28	0.004	0.006	0.017

production. The proposed formula could fast estimate the quantity of nutrient salt which completely satisfied the typical semi-continuous vinegar making. Production cost is quite significant for corporations living, so how to cut the cost is urgent for vinegar manufacturers. The organic nitrogen added in this work was expensive than the inorganic nitrogen. In future researches, inorganic nitrogen will be utilized to alter the nitrogen source component to save the production cost.

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