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ORIENTAL JOURNAL OF CHEMISTRY
An International Open Free Access, Peer Reviewed Research Journal

www.orientjchem.org

ISSN: 0970-020 X
CODEN: OJCHEG
2015, Vol. 31, No. (1):
Pg. 581-590

FED-Batch Fermentation For Propionic, Acetic And Lactic Acid Production

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http://dx.doi.org/10.13005/ojc/310174

(Received: September 10, 2014; Accepted: October 15, 2014)

ABSTRACT

Propionibacterium is capable of producing many important industrial products such as propionic acid, vitamin B_{12} and bacteriocin. Also it shows some probiotic health benefits and produces stimulator of intestinal bacteria. The aim of this research is to evaluate the effect of pH control on biomass, propionic, acetic and lactic acids production in fed-batch system by *Propionibacterium freudenreichii* ssp. *shermanii* and *Lactobacillus acidophilus*. Fermentation was conducted in a 3-L fermentor containing base medium and molasses as the carbon source in which milk feeding were added in 36th hour and maintained at 30 °C. Total fermentation time was 144 h. Every 24 h sampling has been done to measure the biomass and organic acids. Yield of biomass and acid production were compared in two separate trials: one treatment without pH control and another in which pH was maintained at 6.5 by using NaOH 1N. Content of biomass and organic acid were measured by freeze drying method and HPLC, respectively. The final concentration of the obtained responses in treatments with and without pH control is as following (g/L): biomass 6.22±0.04 and 13.76 ±0.04; propionic acid, 5.25±0.02 and 5.67±0.01; acetic acid 5.86±0.02 and 6.33±0.06; and lactic acid 11.34±0.09 and 7.40±0.07. In treatment which pH has not been controlled, production of dried biomass, propionic and acetic acid were higher than controlled treatment. So production of a beverage containing propionic acid could be recommended due to satiety-inducing and saturation effect in consumer.

Key words: *Propionibacterium freudenreichii* subsp. *Shermanii*, propionic acid, fed-batch system, carbon source, molasses, fermentation.

INTRODUCTION

Propionibacterium is gram-positive, nonmotile, catalase-positive, nonsporeforming, rod-shaped and anaerobic to aerotolerant bacteria.[1] The genus of Propionibacterium is separated into two groups: the "cutaneous" and the "dairy" Propionibacteria, based on their habitat.[2] They also may contribute to natural fermentations of silage and olives; and can produce a variety of industrially integral products such as propionic acid, vitamin B_{12}, and bacteriocins. Recently, propionic acid bacteria have gained much attention as both probiotics beneficial for human health and producers of prebiotics selectively stimulating the growth of beneficial intestinal bacteria such as bifidobacterial species.[3]

Propionibacterium simulates growth of Bifidobacterium as probiotic and in this way, regulate microbial flora and cause digestive health. In addition, these bacteria prevent activities of enzymes which producing mutagenic agents and thereby boost the immune system.[4] They effect on intestinal pH through the production of short-chain fatty acids and increase the absorption of iron and calcium.[5,6] Among produced short-chain fatty acids (acetic, propionic and butyric acids) have significant role in induce satiety[7] by simulate secretion of intestinal peptide YY as appetite suppressants[8,9,10] and delayed gastric emptying due to the production of short-chain fatty acids, especially propionic acid.[11] Propionibacterium can reduce plasma cholesterol levels by inhibition of cholesterol synthesis in liver via inhibition of enzyme activity of hydroxy methyl glutaryl CoA synthase and increase fecal excretion of bile acids and cholesterol intake to re-synthesis of bile acids.[12,13] Some strains of Propionibacterium are able to produce vitamins such as B_2 and B_{12}.[14,15]

Studies show that the optimal conditions for the growth of Propionibacterium species are 30-37 °C and pH 6 to 7.[16] Optimum pH range is between 4.6 to 5.8. Below than pH of 4.5, growth will be stopped and acid production will be reduced, so higher inoculum size require for growth.[17] Propionibacterium are able to use different carbon sources such as glucose,[18,19] maltose,[20] sucrose,[21] lactose,[22,23] lactate[19,24] and glycerol.[25,26] It can also use complex sources such as hemicelluloses, corncob molasses and sugarcane molasses and etc.[27]

Since, the application of conventional and expensive systems of fermentation is limited due to the low concentration, yield and productivity, the increased yields of propionic acid obtained by fermentation of cheap industrial waste e.g. glycerol or renewable sources e.g. molasses, biological production can be economically justified.[16,28] Molasses is a renewable resource as a waste of sugar factories. This complex carbon source is able to produce large quantities of biomass and can be used where high cell mass production is concerned. For instance, the production of secondary metabolites such as vitamin B_{12} can initially reach plenty of biomass with consumption of molasses by the microorganism that is useful for extracting vitamin B_{12}.[1]

The major problem of batch system is strong inhibitory of final product on production yield, slow growth of bacteria[29,30] and difficulty of extraction from media.[31] Other processes, including multi-stage,[32] cell immobilization,[33] using fed-batch[24] and continuous culture system[34] have been used to increase yield of propionic acid production.[24] In fed-batch system a simple feeding strategy was used for the supplementation of nutrient e.g. sugar at frequent intervals and constant feed rate. Feeding may be start when the growth rate is high, to eliminate nutrient depletion and avoid the accumulation of inhibitory products.[22] Few studies have assessed propionic acid production by fed-batch fermentation model and the use of molasses as a carbon sources. So, there is limited evidence of study on the effect pH on the organic acid production by Propionibacterium with use of molasses as a carbon sources. If the purpose of using Propionibacterium is a health benefit in terms of a functional food, use of NaOH is an important issue while controlling pH; that requires study on its effects on organic acids production. In our previous research, maximum propionic acid was achieved by inoculation rate of 1:4 *Lactobacillus acidophilus* and *P. freudenreichii* spp *shermanii*.[35] In this study, the attempt scale up of production, change of

fermentation system from flask to 1.2 L bioreactor and study effect of pH control on propionic. Acetic and lactic acids production by mentioned inoculums.

MATERIAL AND METHODS

Microorganisms and Inoculums Preparation

P. freudenreichii ssp. *shermanii* DSM 20270 and *L. acidophilus* LA5 were obtained from IROST (Iranian *Research Organization* for Science and Technology). *P. freudenreichii* was grown in Propionibacterium culture (composition: 1% pancreatic digests of casein, 0.5% yeast extract, and 1% sodium lactate) and was incubated anaerobically for 48 h at 30 °C.

The conservation medium held per liter of deionized water: 1 g KH_2PO_4, 2 g $(NH_4)_2HPO_4$, 2.5 mg $MnSO_4 \cdot H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, 10 mg $MgSO_4 \cdot 7H_2O$, 10 mg $CaC_{l2} \cdot 6H_2O$, 10 mg $CoC_{l2} \cdot 6H_2O$, 5.0 g yeast extract, 5.0 g sodium lactate, and 7.0 g agar, and pH was adjusted to 6.8 before autoclaving. The preculture and the inoculum media had the same composition as the conservation medium minus agar. In addition, sodium lactate concentration was increased to 20 g/L, whereas yeast extract concentration was increased to 10 g/L. One separated colony from deep agar plate was transferred to 2 mL of preculture medium and incubated at 30 °C for 48 h.

A portion of this culture (0.4 mL) was transferred to 40 mL screw-cap flask holding 40 mL of inoculum medium broth. *P. freudenreichii* was grown without agitation for 24–36 h at 30 °C in inoculum medium broth and was inoculated at 1% (v/v), into 1.2 L of fermentation broth in the 3-L fermentor. The cell count of the pre-inoculums was 4.2×10^9 CFU m/L.

Fed-batch Fermentations

The fermentation medium for fed-batch fermentation had 25 g molasses [basal medium with sugarcane molasses (BMSM)] and 350 mL skim milk (contains ~ 23 g lactose) was added as feeding source. The basal medium and the carbon sources were prepared independently. The pH of these two solutions was adjusted to 6.5±0.05 before autoclaving.

Skim milk powder was diluted with 300 mL distilled water in a bottle and was sterilized at 121° C and 1 bar for 15 s. The fed-batch system was adjusted for 144h fermentation at 30° C and feeding was started after 36 hours for 8 hours by constant speed of 0.03 L/h. Two treatments were compared to each other; in one medium pH was maintained at 6.5 by using NaOH 1N, but in another series, during fermentation pH wasn't controlled and decreased. Samples of 20 mL were removed each 24 h of the fermentation.

Biomass Determination

After sampling from fermentor, 20 mL of samples was centrifuged at 12000×g for 10 min at 4 ÚC. The supernatant phase was washed and was added to a plate for freeze-drying. Plates were weighed after freeze-drying.

Organic Acid Determination

As described before (Farhadi et al., 2012) separation and quantification of propionic, lactic and acetic acids was done using a High Performance Liquid Chromatography (CE 4200, Cecil, Milton Technical Center, Cambridge, UK). In a short period, for extraction of acids, 6 ml of sample was diluted into 5 mL of 0.5 N H2SO4. After centrifugation (at 5000×g for 15 min), 3 mL of upper phase was filtered through 0.45-¼m Gelman Acrodisc filters and injected into HPLC system.

The chromatographic system contains two CE-4200 Dual piston pump, one CE-4200 UV visible detector, a vacuum degasser and a dynamic mixing chamber. An Agilent technologies (Palo Alto, CA, USA) Eclipse C_{18} column (4.6 mm × 250 mm i.d, 5 ¼m) was used for separation. The mobile phase was a binary solvent with constant ratio (30:70) of methanol: water (adjusted by sulphuric acid 5×10^{-4} M) with total flow rate of 1 mL/min. The volume of injection loop was 20 ¼L. The detection wavelength was set at 210 nm and the analysis was carried out at ambient temperature. All experiments were performed in triplicate. The standard solutions of propionic, lactic and acetic acids (Merck, Darmstadt, Germany) were prepared in distilled water. Initial identity assignment of organic acids (propionic, lactic and acetic acids) was based on comparison of retention data gained with the UV detector for standard compounds and

sample components. Quantification was accomplished using peak areas from external calibration with standard solutions.

RESULTS

pH changes during fermentation

Figure 1 shows pH profile during microbial production process. In fed-batch pH was maintained at 6.5 by automatic adding 1 N NaOH in treatment 1 while it's allowed to drop in pH in treatment 2 and final pH was reached to 4.6. As can be seen in Figure 1, pH drop is a significant amount from the starting time until feeding time and this dropping continues by adding secondary carbon sources (lactose as a feed), but by reaching the end of fermentation, this decreasing is very small. It can be seen that bacteria are able to use carbon sources quickly during the initial 36 hours and rapid production of acids which causes pH dropping.

Dried biomass changes during fermentation

Change of dried biomass in both different treatments is shown in figure 2. It is observed that the concentration of produced biomass in the first 48 hours is pretty fast in both treatments. Then the growth rate is almost constant without pH control. The concentrations for both treatments with and without pH control were 6.19±0.10 and 13.76±0.04 g/L, respectively. Differences between these

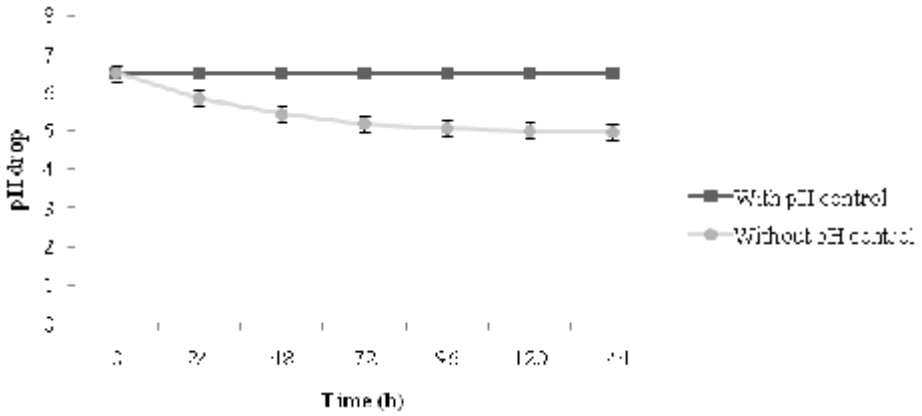

Fig. 1: pH changes during fermentation in two treatments; closed squares are related to treatment 1 (fermentation with pH control by adding 1 N NaOH) and closed circles are related to treatment 2 (fermentation without pH control)

Table 1: pH variation and dried biomass concentration at two different treatments during fed-batch fermnentation by molasses and lactose as carbon sources after 144h

Time (h)	Treatment (T_1* and T_2**)	pH variations	Dried biomass concentration (g/L)	P_{value}
24	T_1T_2	6.505.85	3.65±0.052.44±0.60	0.002
48	T_1T_2	6.505.44	7.33±0.068.44±0.05	0.003
72	T_1T_2	6.505.19	6.62±0.048.71±0.07	0.001
96	T_1T_2	6.505.07	5.56±0.039.57±0.05	0.000
120	T_1T_2	6.505.00	6.15±0.0711.72±0.03	0.000
144	T_1T_2	6.504.97	6.22±0.0413.76±0.04	0.000

*T_1 = Treatment 1 (fermentation with pH control by adding 1 N NaOH)
**T_2= Treatment 2 (fermentation without pH control which allow to drop in pH

treatments statistically are significant (P<0.005) and biomass production during fed-batch fermentation without pH controlling is better than another one.

Organic acid changes during fermentation

Propionic acid production is rather fast at initial time of fermentation and after 48 h the trend of propionic acid production in both treatments is similarly increasing. It is noted that propionic acid concentration in treatment 2 is more than treatment 1 (P<0.05). Acetic acid changes during fermentation time are very similar in both treatments; at the beginning, acid production in treatment 1 is faster. However, at the end of fermentation process, acid concentration in treatment 2 is more than treatment 1 (P<0.05). It has been observed that lactic acid production was decreased in pH control significantly more (P<0.05) and this difference is more pronounced after 72 h.

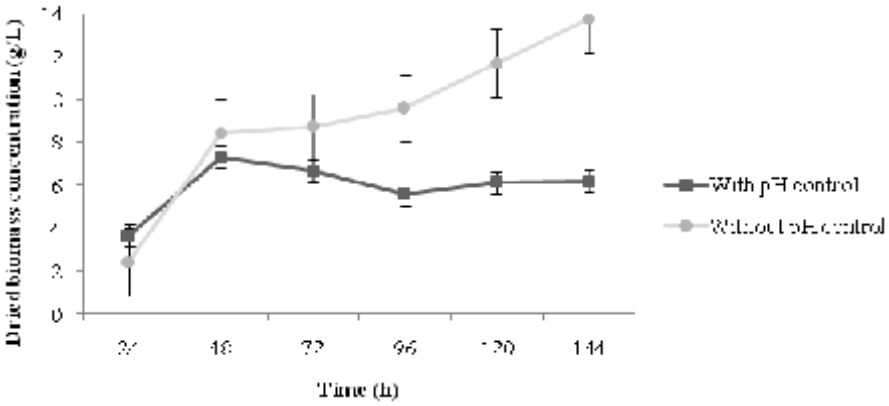

Fig. 2: Dried biomass changes during fermentation in two treatments; closed squares are related to treatment 1 (fermentation with pH control by adding 1 N NaOH) and closed circles are related to treatment 2 (fermentation without pH control)

Table 2: Propionic, acetic acid and lactic acid concentration at two different treatments during fed-batch fermnentation by molasses and lactose as carbon sources after 144h

Time (h)	Treatment (T_1* and T_2**)	Propionic acid conc. (g/L)	Propionic acid P_{value}	Acetic acid conc. (g/L)	Acetic acid P_{value}	Lactic acid conc. (g/L)	Lactic acid P_{value}
24	T_1	1.87±0.04	0.000	3.95±0.04	0.000	Not detected	0.000
	T_2	0.25±0.04		Not detected		2.17±0.03	
48	T_1	3.16±0.04	0.028	4.27±0.03	0.012	9.04±0.05	0.268
	T_2	3.36±0.02		4.01±0.08		8.95±0.05	
72	T_1	3.32±0.03	0.000	4.56±0.02	0.478	9.23±0.05	0.038
	T_2	4.26±0.00		4.61±0.08		8.92±0.05	
96	T_1	3.82±0.01	0.000	4.87±0.06	0.293	9.61±0.08	0.000
	T_2	4.45±0.03		4.80±0.03		8.19±0.04	
120	T_1	4.29±0.01	0.001	5.18±0.03	0.010	10.93±0.02	0.000
	T_2	4.91±0.02		5.52±0.05		7.46±0.07	
144	T_1	5.25±0.02	0.002	5.86±0.02	0.10	11.34±0.09	0.000
	T_2	5.67±0.01		6.33±0.06		7.40±0.07	

*T_1 = Treatment 1 (fermentation with pH control by adding 1 N NaOH)
**T_2= Treatment 2 (fermentation without pH control which allow to drop in pH)

Vitamin B_{12} production

The final concentration of vitamin B_{12} in fermentation broth at treatment 1 was 3.9±0.01 mg/L while its concentration at treatment 2 was 0.05±0.00 mg/L. Despite more biomass production at treatment 2, vitamin production as a secondary metabolite is less in treatment 2 in compression with treatment 1.

It was observed that final concentration of dried biomass (6.22±0.04 g/L) and both organic acids, propionic (5.25±0.02 g/L) and acetic

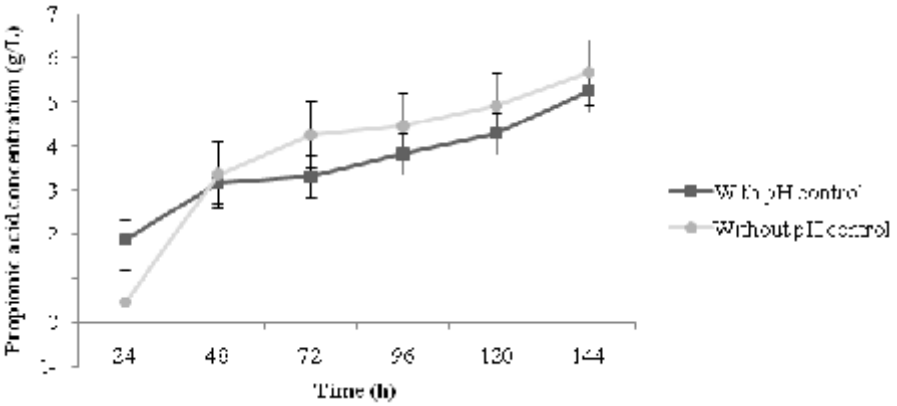

Fig. 3: Propionic acid changes during fermentation in two treatments; closed squares are related to treatment 1 (fermentation with pH control by adding 1 N NaOH) and closed circles are related to treatment 2 (fermentation without pH control)

Table3. Effects of pH on propionic, acetic and lactic acid fermentation related to productivities at two different treatments during fed-batch fermnentation by molasses and lactose as carbon sources after 144h

Time (h)	Treatment (T_1* & T_2**)	[a]P_X (mg/L)	P_X P_{value}	[b]P_P (mg/L)	P_P P_{value}	[c]P_A (mg/L)	P_A P_{value}	[d]P_L (mg/L)	P_P P_{value}
24	T_1	21.13±0.28	0.001	10.81±0.21	0.000	22.88±0.26	0.000	-	0.000
	T_2	14.13±0.32		1.46±0.25		-		12.55±0.27	
48	T_1	42.40±0.33	0.003	18.27±0.22	0.028	24.72±0.16	0.014	52.34±0.34	0.270
	T_2	48.86±0.31		19.43±0.12		23.20±0.47		51.80±0.28	
72	T_1	38.31±0.26	0.001	19.24±0.18	0.000	26.38±0.11	0.478	53.43±0.32	0.038
	T_2	50.40±0.44		24.67±0.03		26.67±0.47		51.64±0.30	
96	T_1	32.19±0.20	0.000	22.13±0.83	0.000	28.21±0.36	0.293	55.63±0.49	0.002
	T_2	55.37±0.32		25.75±0.20		27.78±0.16		47.41±0.21	
120	T_1	35.61±0.43	0.000	24.81±0.07	0.001	29.99±0.20	0.027	63.26±0.13	0.000
	T_2	67.82±0.17		28.45±0.13		31.60±0.32		43.16±0.40	
144	T_1	36.03±0.22	0.000	30.36±0.12	0.002	32.90±1.64	0.040	65.65±0.54	0.000
	T_2	79.64±0.22		32.28±0.06		36.63±0.38		42.83±0.42	

*T_1 = Treatment 1 (fermentation with pH control by adding 1 N NaOH)

**T_2= Treatment 2 (fermentation without pH control which allow to drop in pH)

(a)dried acid productivity

(b)Propionic acid productivity

(c)Acetic acid productivity

(d)Lactic acid productivity

(5.86±0.02 g/L) in treatment 1, were lower than in treatment 2 (13.76±0.04, 5.67±0.01 and 6.33±0.06 g/L, respectively). But lactic acid final concentration in treatment 1 was 11.34±0.09 g/L which was more than treatment 2 (7.40±0.07 g/L) and for vitamin B_{12}, concentration at treatment 1 significantly is more than another one.

DISCUSSION

Considering the experimental fed-batch fermentations the maximum biomass concentration was obtained without pH control which was higher than that of when pH was controlled. Therefore, NaOH is seen as an inhibitory agent. During fermentation, the amounts of organic acids produced increase so more NaOH is required to hold pH at constant amount. In this condition use of large volume of NaOH may cause negative effect on bacterium growth.

Molasses contains high amounts of sucrose and is suitable for growth of bacteria, particularly Propionibacterium. At the beginning of the fermentation process, the bacteria consume the carbon source to grow rapidly. Lactose was added to fermentation broth, when molasses was finishing. Since lactose is favorable substrate for lactobacillus, bacteria will continue growing by consuming lactose. Continued production of biomass in the absence of NaOH increases even until the end of fermentation. According to increased

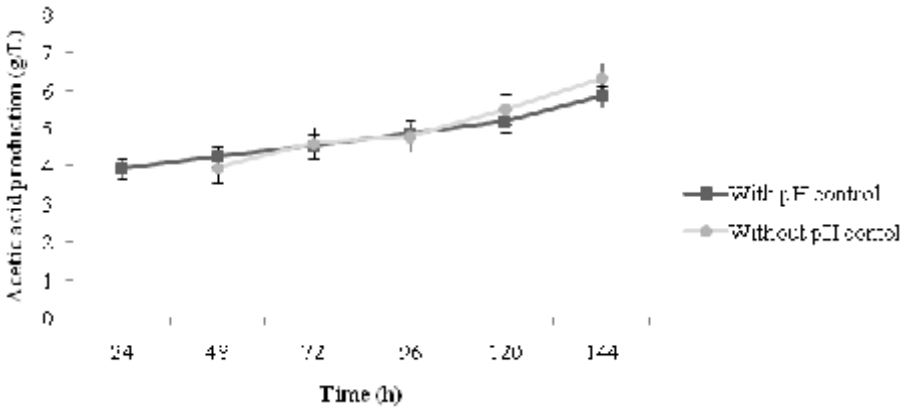

Fig. 4: Acetic acid changes during fermentation in two treatments; closed squares are related to treatment 1 (fermentation with pH control by adding 1 N NaOH) and closed circles are related to treatment 2 (fermentation without pH control)

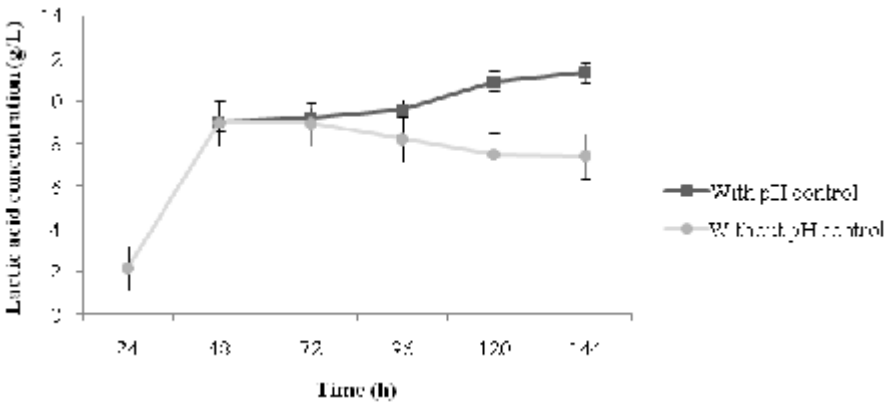

Fig. 5: Lactic acid changes during fermentation in two treatments; closed squares are related to treatment 1 (fermentation with pH control by adding 1 N NaOH) and closed circles are related to treatment 2 (fermentation without pH control)

concentration and productivity of biomass can be concluded that, fermentation without pH control is more suitable for growth of *P. freudenreichii*.

Also, competitive inhibitory effect of acetic acid on propionic acid production was demonstrated. Therefore, during the fermentation by producing more acetic and lactic acid the production of propionic acid was affected due to acid inhibitory effect. By feeding of lactose to culture medium and consequently its consumption by lactobacillus, production of lactic acid was increased. Also propionibacterium are able to use lactic acid as an alternative substrate. Studies show that in fermentation by *P. freudenreichii* 3 moles of lactic acid are converted to 2 moles of propionic acid, 1 mole acetic acid and 1 mole carbon dioxide.[36] So, reason for increased production of two organic acids after 48 hours is consumption of lactic acid by bacteria as a carbon source.

CONCLUSION

Statistical results showed that no pH control has a significant positive impact on propionic and acetic acid production. This becomes more important when consider to nutrition and consumption aspect of broth containing propionic acid. If the purpose of propionic acid production is for nutritional properties in a functional food, lack of NaOH may be more important. Due to the relatively high amount of produced propionic acid in the sample and the higher yield of acid production in run without pH control and also the use of molasses as a cheap abundant carbon source, the results of this research have made it possible to make a functional beverage. However, scrutiny of satiety effect of this product in a one nutritional study is recommended.

According to beneficial role of lactose and *L. acidophilus* for production of propionic and acetic acid, using carbon sources include lactose such as milk is recommended. Moreover, considering the antifungal properties of propionic acid, in dairy product produced by this method with satiety effect, nutritional properties of probiotics and vitamin B_{12}, it has a natural preservative and it helps to do not use any chemical preservatives such as sorbate.

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