

## Stimulation of the *Megasphaera elsdenii*'s Butyrate Production in Continuous Culture by a Yeast Additive

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### ABSTRACT

Continuous culture study of the ruminal bacterium *Megasphaera elsdenii* using lactate as carbon source is reported. Butyrate, the minor product in batch culture, was the main product in continuous culture. Under steady state conditions the biomass concentration was increased from 0.46 to 0.72 g l<sup>-1</sup>. Addition of Yea Sacc did not result an increased availability of carbon. The produced amounts of valerate and CO<sub>2</sub> were calculated by using a combined carbon and degree of reduction balances. According to these results, the lactate catabolism of *M. elsdenii* was changed by the presence of yeast additive, yielding more butyrate and diminishing the production of valerate, while acetate and propionate production was not affected. Results reported in this work suggested that soluble compounds present in the yeast additive filtrate enhanced the anabolism of *M. elsdenii* and modified the carbon fluxes through its pathways by increasing butyrate and decreasing valerate production in continuous culture.

**Key words:** *Megasphaera elsdenii*, butyrate, yeast additive, continuous culture

### INTRODUCTION

*Megasphaera elsdenii*, the main lactate utilizer rumen bacterium, is capable of fermenting up to 97% of the rumen lactate (Counotte *et al.*, 1981). The volatile fatty acids (VFA, acetate, propionate and butyrate) are the main products of lactate metabolism (Marounek & Bartos, 1987; Marounek *et al.*, 1989; Hino *et al.*, 1994). On the other hand, the effect of additives on microbial activity has been reported (Wallace & Newbold, 1992), both growth and lactate uptake are stimulated by the commercial additives: Amaferm (Waldrip & Martin, 1993), Yea Sacc (Rossi *et al.*, 1995) and

Levucell SC (Chaucheyras *et al.*, 1996). Nevertheless, their effect on microbial fermentation patterns are not well known.

Continuous culture of *M. elsdenii* has been utilized to evaluate the effect of pH on the efficiency of growth (Russell & Dombrowsky, 1980), to study the catabolism of amino acids (Wallace, 1986) and to evaluate the role of extracellular acetate in the fermentation of glucose (Hino *et al.*, 1991), but previous studies have not considered production of VFA by *M. elsdenii* in continuous culture with lactate as carbon source. In this work, *M. elsdenii* was cultivated in continuous culture and the effect

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of a yeast additive (Yea Sacc<sup>1026</sup>, AllTech<sup>TM</sup>) was assessed upon fermentation pattern.

## MATERIALS AND METHODS

### Micro-organism and culture medium:

*Megasphaera elsdenii*, Spanish Type Culture Collection (CECT) 390, was conserved in glycerol (30%) at -20°C. The culture medium for batch and continuous culture contained (g l<sup>-1</sup>): lactate (as sodium DL-lactate syrup 60% w/v), 10; casein peptone, 8; yeast extract, 2.8; beef extract, 2.8; cysteine hydrochloride, 0.5; Tween<sup>TM</sup> 80, 1 ml l<sup>-1</sup> and mineral solution, 40 ml l<sup>-1</sup>. The mineral solution contained (g l<sup>-1</sup>): CaCl<sub>2</sub>, 0.2; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2; K<sub>2</sub>HPO<sub>4</sub>, 1; KH<sub>2</sub>PO<sub>4</sub>, 1; NaHCO<sub>3</sub>, 10 and NaCl, 2. pH was fixed to 6.7. Yeast additive was prepared under anaerobic conditions as follows: 4 g of Yea Sacc<sup>1026</sup> (AllTech<sup>TM</sup>) plus 50 ml of deionized water were stirred during 1 hour. The suspension was passed through a filter paper Whatman #41 and sterilized by filtration through a 0.45 µm membrane. The filtered liquid was bubbled with O<sub>2</sub>-free CO<sub>2</sub> during 10 minutes. To study the effect of the yeast additive, the resulting solution was added to culture medium in order to obtain a concentration of 0.675 g l<sup>-1</sup> (2% v/v).

**Reactor operation:** Continuous culture was performed in a B-Braun fermenter model Biostat-B. Culture vessel with 1800 ml of medium was sterilized at 121°C during 1 hour. Oxygen was eliminated with a continuous flow of nitrogen (10 ml min<sup>-1</sup>), which was maintained during all the process in order to preserve anaerobic conditions. Medium was inoculated with 200 ml of an overnight culture of *M. elsdenii* CECT 390 and the fermentation was carried out at 37°C and 100 min<sup>-1</sup>. The system was first operated in batch culture during 18 h. After this time, continuous culture was started at a dilution rate of 0.1 h<sup>-1</sup>. Two stages in continuous culture were studied. In the first stage, medium without additive was used. Later, medium plus yeast additive was supplied during second stage.

### Sample analysis and expression of results:

Turbidimetric (Goodhue *et al.*, 1986), enzymatic (Boehringer Mannheim, 1999) and gas chromatography (Jouany, 1982) techniques were used for biomass, DL-lactate and VFA

measurements, respectively. Measured acetate, propionate and butyrate were expressed individually and as total VFA. Biomass (Y<sub>XS</sub>), acetate (Y<sub>AS</sub>), propionate (Y<sub>PS</sub>) and butyrate (Y<sub>BS</sub>) yields were calculated as indicated by Stephanopoulos and coworkers (1998). Units of these yield coefficients are given in Table 1, and expressed in terms of one C-mole.

## RESULTS AND DISCUSSION

### Growth and lactate consumption

Biomass, DL-lactate and VFA evolution during the process are shown in Figure 1, where the batch and two stages of continuous culture can be observed. During batch culture, a maximum biomass concentration of 0.75 g l<sup>-1</sup> was obtained. This result is in agreement to that obtained by Rossi *et al.* (1995) working with a medium of similar composition. Fig. 1a shows that biomass concentration was kept constant (0.46±0.01 g l<sup>-1</sup>) during 80 hours of continuous culture and therefore a steady state was considered. In order to simulate a step type perturbation, enough solution of Yea Sacc was added to the culture to obtain the concentration of Yea Sacc indicated previously (0.675 g l<sup>-1</sup>). Then, the culture was fed with a broth containing the yeast additive (0.675 g l<sup>-1</sup>). Concentration of biomass increased until a new steady state (0.72±0.03 g l<sup>-1</sup>, during 50 hours), as indicated in Figure 1a. Biomass concentration increased 54% in the presence of the yeast additive. A Student *t* test indicated significant difference (p<0.05) between steady state concentrations of biomass.

If complete utilization of yeast additive (0.675 g l<sup>-1</sup>) as carbon source was assumed and considering the highest reported yield of biomass (0.25 g g<sup>-1</sup>, for glucose at D ~ 0.1 h<sup>-1</sup>) for *M. elsdenii* (Russell and Baldwin, 1979), it explained up to 68% of the observed biomass increase. Then, biomass concentration increase could not be explained by a yield related to the used amount of additive and Yea Sacc filtrate did not mean an increased availability of carbon.

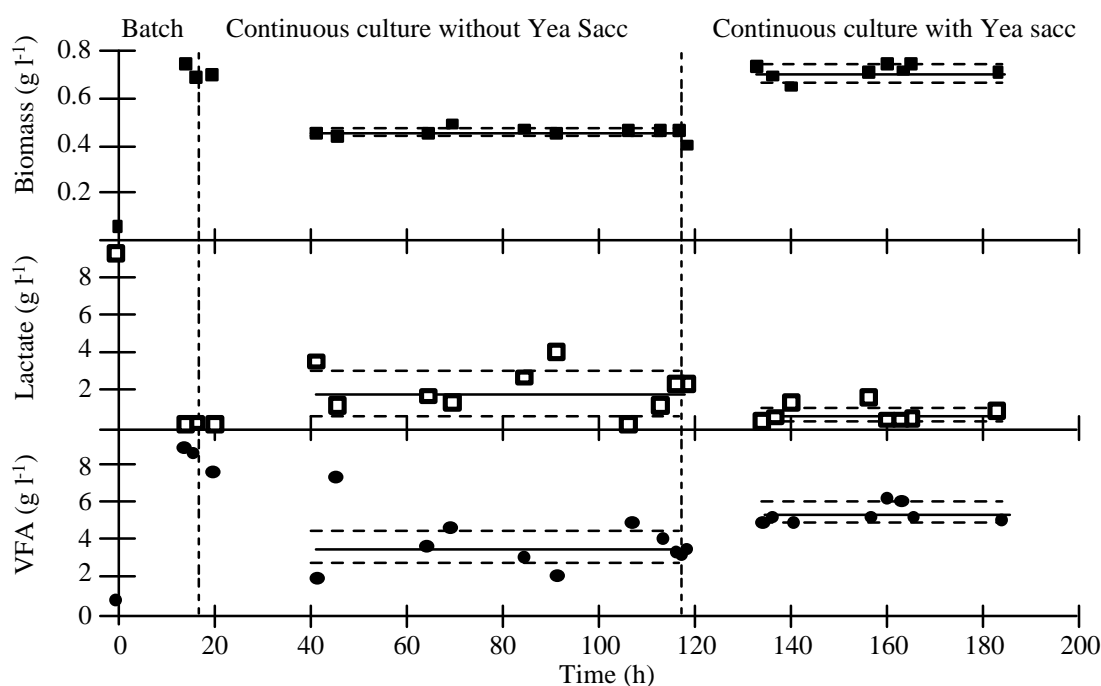
Lactate concentration in the feed was 9.32±0.21 g l<sup>-1</sup> along of continuous culture. Residual concentration of lactate during steady state without additive was 1.84±1.03 g l<sup>-1</sup> (Figure 1b), then the

consumed amount of lactate was  $7.48 \pm 0.82 \text{ g l}^{-1}$  in this stage. In the second steady state (in the presence of the yeast additive), the consumed amount of lactate was  $8.63 \pm 0.26 \text{ g l}^{-1}$  (residual concentration,  $0.69 \pm 0.47 \text{ g l}^{-1}$ ). According to this when the yeast additive was present, a greater consumption of lactate was observed. Steady state DL-lactate consumption was increased 18% and

significant differences were found by using a Student *t* test ( $p < 0.05$ ).

#### Volatile fatty acids production

Total VFA concentration increased 37% ( $p < 0.05$ ) when the additive was present in continuous culture (Figure 1c). Butyrate was the minor product in batch culture, but it was



**Figure 1** - Biomass (a), DL-lactate (b) and VFA (c) concentrations during batch and continuous culture of *Megasphaera elsdenii* without and with the presence of Yea Sacc<sup>1026</sup> (AllTech™).

the main product in continuous culture while acetate and propionate proportions were decreased. Hino and coworkers (1991) found that the molar fraction of butyrate was decreased from 0.66 to 0.50 when batch culture was changed to continuous culture, using glucose as carbon source. Nevertheless, in this work it increased from 0.11 to 0.53 while using lactate as carbon source. It would worth to indicate that *M. elsdenii* did not produce propionate during fermentation of glucose (Hino & Kuroda, 1993).

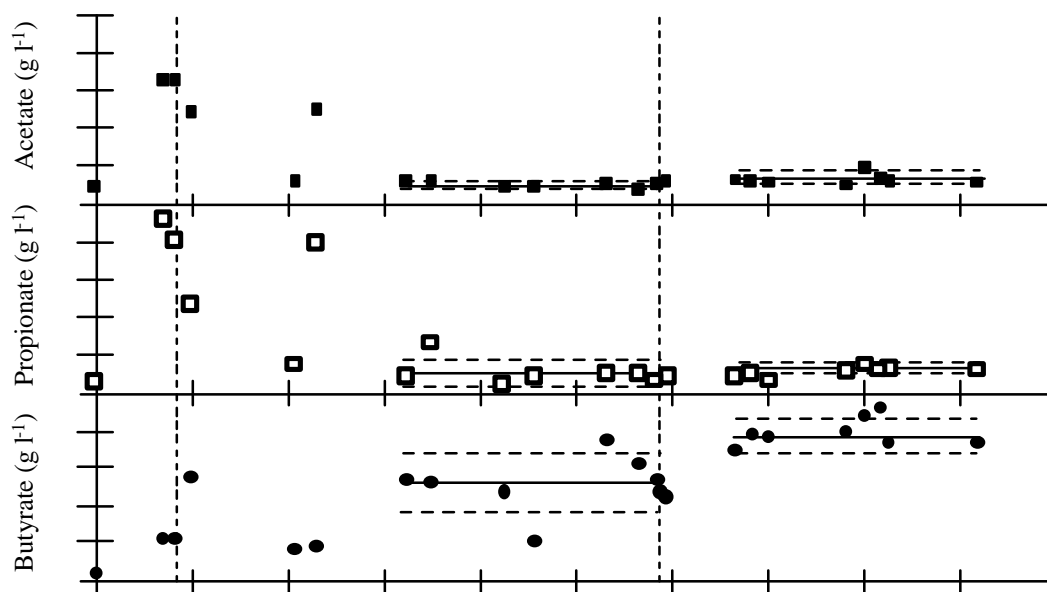
Figure 2 shows the individual evolution of VFA. Concentrations of acetate and propionate decreased during the early stage of continuous culture and remained nearly constant from 40 to 120 h. A Student *t* test did not show significant differences ( $p < 0.05$ ) between steady state concentrations. In fact, the variation of the total concentration of VFA

could be attributed mainly to the variation of butyrate concentration. On the other hand, under steady state conditions yeast additive did not affect the concentrations of acetate and propionate, but increased the level of butyrate. A Student *t* test indicated significant difference ( $p < 0.05$ ) between steady state concentrations of butyrate when yeast additive was added. This result was different from that reported by Callaway & Martin (1997), who found that a yeast additive increased the production of acetate and propionate and did not change the butyrate production, working in batch culture and using a different yeast additive (Diamond V XP™). Table 1 presents the yields of biomass and formed products, where all species present in the fermentation are expressed on the basis of one C-mole. Values in this table showed that yields of acetate and propionate were not changed by the

presence of the yeast additive, while biomass and butyrate yields were increased. In each stage of continuous culture, all yields were added and addition should be equal to one, in order to obtain a carbon balanced fermentation. The addition of yields for steady states were not equal to one, then other products must be formed. On the other hand, according to actual knowledge of the metabolism of

*M. elsdenii* (Gottschalk, 1986; Marounek *et al.*, 1989) valerate and CO<sub>2</sub> must have been also produced under experimental conditions used. Metabolic engineering fundamentals indicate that in a black-box model carbon balance:

$$0=1-Y_{XS}-Y_{AS}-Y_{PS}-Y_{BS}-Y_{VS}-Y_{CS} \quad (1)$$



**Figure 2** - Acetate (a), propionate (b) and butyrate (c) concentrations during batch and continuous culture of *Megasphaera elsdenii* without and with the presence of Yea Sacc<sup>1026</sup> (AllTech<sup>TM</sup>).

and degree of reduction balance:

$$0=\gamma_S-\gamma_X Y_{XS}-\gamma_A Y_{AS}-\gamma_P Y_{PS}-\gamma_B Y_{BS}-\gamma_V Y_{VS} \quad (2)$$

must be satisfied (Stephanopoulos *et al.*, 1998). Variables in equations (1) and (2) are:

$Y_{VS}$  = Yield of valerate

$Y_{CS}$  = Yield of CO<sub>2</sub>

$\gamma_S$  = Degree of reduction of lactate = 4

$\gamma_X$  = Degree of reduction of biomass = 4.14

$\gamma_A$  = Degree of reduction of acetate = 4

$\gamma_P$  = Degree of reduction of propionate = 4.67

$\gamma_B$  = Degree of reduction of butyrate = 5

$\gamma_V$  = Degree of reduction of valerate = 5.2

Biomass degree of reduction was calculated for an average biomass composition (Stephanopoulos, *et*

*al.*, 1998). It must be noted that CO<sub>2</sub> did not participate in the degree of reduction balance (equation 2), because it is considered redox neutral. Solving to unknown values of  $Y_{VS}$  and  $Y_{CS}$  for the steady state without additive:

$$Y_{VS} = 0.148 \text{ (mol C-valerate) (mol C-lactate)}^{-1}$$

$$Y_{CS} = 0.176 \text{ (mol C-CO}_2\text{) (mol C-lactate)}^{-1}$$

In similar way for the steady state with Yea Sacc:

$$Y_{VS} = 0.001 \text{ (mol C-valerate) (mol C-lactate)}^{-1}$$

$$Y_{CS} = 0.165 \text{ (mol C-CO}_2\text{) (mol C-lactate)}^{-1}$$

According to these results, the lactate catabolism of *M. elsdenii* was changed yielding more butyrate and diminishing the production of valerate, while acetate and propionate production was not affected. The effect of some additives on the growth and

lactate uptake by *M. elsdenii* has been mainly studied in batch culture. In this work data of continuous culture by *M. elsdenii* indicated that biomass and butyrate production were stimulated due to the presence of Yea Sacc filtrate.

*M. elsdenii* is the main lactate utilizer in the rumen (Counotte *et al.*, 1981), and has a very important

role to prevent lactic acidosis (Kung & Hession, 1995). Results presented here suggested that Yea Sacc filtrate could contain growth factors that improved the growth of *M. elsdenii*, increasing the  $Y_{XS}$  from 0.071 to 0.095 in the presence of additive, and enhancing its capacity to diminish the lactate concentration in the medium. On the other

**Table 1-** Effect of Yea Sacc<sup>1026</sup> (AllTech<sup>TM</sup>) on the yields of biomass and formed products in continuous culture by *Megasphaera elsdenii*.

	Without Yea Sacc	With Yea Sacc
$Y_{XS}$ [(mol C-biomass) (mol C-lactate) <sup>-1</sup> ]	0.071	0.095
$Y_{AS}$ [(mol C-acetate) (mol C-lactate) <sup>-1</sup> ]	0.066	0.070
$Y_{PS}$ [(mol C-propionate) (mol C-lactate) <sup>-1</sup> ]	0.076	0.072
$Y_{BS}$ [(mol C-butyrate) (mol C-lactate) <sup>-1</sup> ]	0.463	0.597
$Y_{VS}^*$ [(mol C-valerate) (mol C-lactate) <sup>-1</sup> ]	0.148	0.001
$Y_{CS}^*$ [(mol C-CO <sub>2</sub> ) (mol C-lactate) <sup>-1</sup> ]	0.176	0.165

\*Estimated by degree of reduction and carbon balance in a black-box model.

hand, Dawson and coworkers (1990) pointed out that live yeast cells were necessary in the rumen bacteria growth stimulation. Our results suggested that soluble compounds in the filtrate without living cells have an important effect. Callaway & Martin (1997) reported similar results for *M. elsdenii* growing in batch culture. Their results suggested that soluble factors in the filtrate were involved in stimulating growth.

Results reported in this work suggested that soluble compounds present in the yeast additive filtrate enhanced the anabolism of *M. elsdenii* and modified the carbon fluxes through its pathways, by increasing butyrate and decreasing valerate production in continuous culture.

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## RESUMO

Estudo em cultura contínua da bactéria ruminal *Megasphaera elsdenii* que utiliza lactato como fonte de carbono foi realizada. Butirato, foi o produto produzido em menor concentração na cultura em batelada, porém em cultura contínua foi o produto de maior concentração.

Sob condições definidas a concentração de biomassa aumentou de 0.46 a 0.72 g l<sup>-1</sup>. A adição do aditivo Yea Sacc não resultou num aumento da disponibilidade do carbono. As quantidades produzidas de valerato e CO<sub>2</sub> foram calculadas pelo uso combinado do balanço e grau de redução do carbono. De acordo com esses resultados, o catabolismo de lactato de *M. elsdenii* foi mudado pela adição de aditivo a base de levedura. Maior rendimento em butirato e redução na produção de valerato foi observada, por outro lado a produção de acetato e propionato não foi afetada. Os resultados desse trabalho sugerem que os componentes solúveis presentes no aditivo a base de levedura aumentam o anabolismo de *M. elsdenii* modificando o fluxo de carbono através de suas vias de metabólicas, pelo aumento do butirato e redução do valerato. Em cultura contínua.

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