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Propagation of *Streptomyces aureofaciens* MY18 in the Fermentor as Batch and Continuous Cultures

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ABSTRACT. *Streptomyces aureofaciens* MY18 (local isolate) was grown in the fermentor as batch and continuous chemostat cultures. The constant and suitable environment in batch cultures increased biomass and antibiotic productivity by this strain. Respiratory quotient was approximately constant, whereas O_2- uptake and CO_2 production increased gradually to reach their maximum on 7*th* day of incubation. Continuous cultivation at different dilution rates revealed that the highest efficiency in the utilization of added nutrients was achieved at 0.0255 h^{-1} (25.5 ml h^{-1} flow rate). The steady state of this dilution rate gave constant values of starch consumed, starch utilization efficiency, accumulation of antibiotics, O_2- uptake and CO_2 production. The increase in dilution rate to 0.0321 h^{-1} (32.1 ml fresh medium h^{-1}) caused general decreases of all biological activities leading to the phenomenon of washing out. Optimum pH in chemostat culture was 7, and increase or decrease of pH than this level decreased respiration rate, and, consequently, the production of biomass and antibiotic was reduced. The crude antibiotic extracted by *n*-butanol from the culture filtrate gave a considerable antibiotic activity as compared to tetracycline.

Introduction

It is very difficult to cultivate microorganisms in shaken cultures under constant conditions, such as pH and dissolved oxygen which abruptly change during the time course of incubation[1,2]. The growth and biological activity are highly affected by these factors. In the fermentor, pH is continuously controlled by automatic addition of acid or alkaline to keep it constant during propagation. Dissolved oxygen is also controlled to a constant level using a suitable flow rate of air[3]. It means that propa-

gation in the fermentor usually occurs under a constant environment as compared to shaken cultures[4].

In batch cultures, the medium becomes depleted of nutrients at the end of exponential phase, in addition to the accumulation of metabolic products. This led ultimately to a decrease in the rate of growth with the result that the culture enters the stationary phase[5]. In continuous cultures, fresh medium is added to the culture at a rate sufficient to maintain the culture population density at a constant level. A part of the culture is withdrawn automatically at a rate equal to the flow rate of medium addition. This condition prevents the ceasing of growth as it does in a batch culture[6].

The aim of this investigation was to study the biological activities of *Streptomyces aureofaciens* MY18 (local isolate) in the fermentor as batch and continuous cultures with special reference to antibiotic productivity. Biomass, nutrient uptake, oxygen uptake, CO_2 production and growth parameters as influenced by different dilution rates were also investigated.

Material and Methods

Microorganisms Used

Streptomyces aureofaciens MY18 was used throughout this investigation as an antibiotic producer. This strain was isolated by Malibari[7] from the soil of Western region of Saudi Arabia. *Staphylococcus aureus* 209P was used to determine the antibiotic activity of the culture filtrate. Both organisms were obtained from the Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah.

Maintenance of Cultures

The cultures were maintained by lyophilization at –50°C using freeze drier (Labconco). Slants of cultures were also maintained at 4 to 6°C after their propagation on the specific medium for each organism; (Glycerol) casein medium[8] for *Streptomyces* strain; and *Staphylococcus* medium[9] for *Staph. aureus.*

Cultivation in the Fermentor as a Batch Culture

The propagation of *Streptomyces* MY18 was carried out in a Gallenkamp Modular fermentor consisting of 2 litre fermentation vessel equipped with stirrer and aeration device, automatic pH controller, automatic temperature control, and automatic sensing defoament addition control. The fermentation vessel and accessories were autoclaved at 120°C for 20 minutes. One litre of sterile starch nitrate medium[10] was added to the fermentation vessel, in addition to 20 ml of standard inoculum. The aeration was 600 ml/minute. Temperature was automatically kept at 30°C. Flowing air from fermentor was connected with infra-red CO_2 analyzer. Dissolved oxygen was determined using oxygen electrode connected with an oxygen meter; it was adjusted at 100% saturation at the start of propagation. Foams were controlled by sterile polypropylene glycol (2025) using automatic foam control system. During batch cultivation, the pH was maintained at 7.0 by addition of NaoH (1*N*) or HCl (1*N*) by means of the automatic pH controller. During batch cultivation, samples (10-20 ml)

were withdrawn from the culture (fermentation vessel) every 24 hours. The samples were filtered, and the pellets were washed twice with distilled water, using mellipore filters, dried at 70°C for two days, and weighed. The culture filtrate was used to determine the consumed starch and effectiveness of excreted antibiotics.

The biomass (dry wt) was plotted against time (days) on semilog paper to determine different growth phases. Growth parameters, *viz.*, specific growth rate, doubling time, and effective yield, were calculated[2,11]. Yield factor (amount of dried biomass per 100 g of starch consumed), and starch utilization efficiency (amount of starch consumed per 100 g of original starch in the medium) were also determined.

Continuous Chemostat Propagation of Streptomyces MY 18

The propagation of the strain under investigation as continuous culture was carried out in the same fermentor described before. After sterilization of the fermentor vessel, addition of starch nitrate medium[10] and 20 ml of standard inoculum were carried out. All accessories (medium reservoir, product reservoir, and medium flow rate pump) of batch and continuous cultures were connected, and the culture was allowed to grow up as a batch culture for 5 days (the end of exponential phase of growth), then fresh medium (starch nitrate medium) was pumped to the culture at the following flow rates (F = ml medium/h): 6.35, 10.00, 19.29, 25.50 and 32.10 ml/h.

These flow rates give the following dilution rates :

(D = F/working volume): 0.00635, 0.01, 0.01929, 0.02550 and 0.03210 h^{-1}. The culture in the vessel was pumped automatically to the culture reservoir at the same rate of medium addition (flow rate) to keep the level of the culture in the fermentor (one litre). Samples were taken at each steady state (resulted from each dilution rate) to determine the biomass and effectiveness of excreted antibiotics. Growth parameters were also calculated as mentioned in batch cultures.

Standard Inoculum of Streptomyces Isolates

Standard inoculum of *Streptomyces* isolates for batch and continuous cultivation was obtained by the propagation of test isolates in glycerol casein medium for 10 days on a rotary shaker (180 rpm) at 30°C. The formed pellets were washed twice with sterile tap water. Pellets were re-suspended in sterile water to form 5-7 pellets per 20 ml, and were used as a standard inoculum for batch and continuous cultures.

Antibiotic Activity

It was determined in the fermented liquor using culture filtrate technique[12]. *Staphylococcus aureus* was used as a sensitive organism.

Starch Consumed

Starch consumed was determined by the method described by Herbet *et al.*[13]

Dissolved Oxygen

It was determined in the air saturated medium according to Taras *et al.*[14].

Carbon Dioxide

Formed amounts of carbon dioxide outlet from the fermentor at certain flow rate was determined using Infra Red Gas Analyzer (IRGA). It was calculated by the following equation :

Amount of CO_2 (ml CO_2 / hour / litre culture) = $C.J.10^{-6}$

where, C = concentration of CO_2 in ppm as indicated by IRGA
J = flow rate of air

The amount of CO_2 produced in *m*mole/h/1 was calculated on the basis that one mole of ideal gas at 0°C and 1 atmosphere = 22.4 litre of this gas. In this work, 1 mole CO_2 = 24.86 litre CO_2 where incubation temperature was 30°C (pressure × volume × absolute temperature^{-1} = constant).

Extraction of Antibiotic

The culture filtrate, either in batch of continuous cultures, was adjusted to pH 2.0 to precipitate the protein[15]. After removing of precipitate, the filtrate adjusted to pH 8 was treated with *n*-butanol and mixed thoroughly in a separating funnel. The *n*-butanol layer was evaporated under vacuum to obtain a crude solid antibiotic. The crude antibiotic was then dissolved in distilled water, and the solution was filtered. The filtrate was lyophilized at –50°C under vacuum using lyophilizer (Labconco; Freeze Dryer 4.5). The residue was redissolved in acetone which was then evaporated under vacuum. The weight of crude antibiotic extracted with butanol water and acetone was then determined. The activity of the antibiotic treated by these solvents was also tested. It was also found valuable to compare the activity of this antibiotic with pure tetracycline (Hostacycline – Hoechst) using different concentrations.

Statistical Analysis

Regression analysis, correlation coefficient (R) and least significant differences (L.S.D.) of the data were calculated[16].

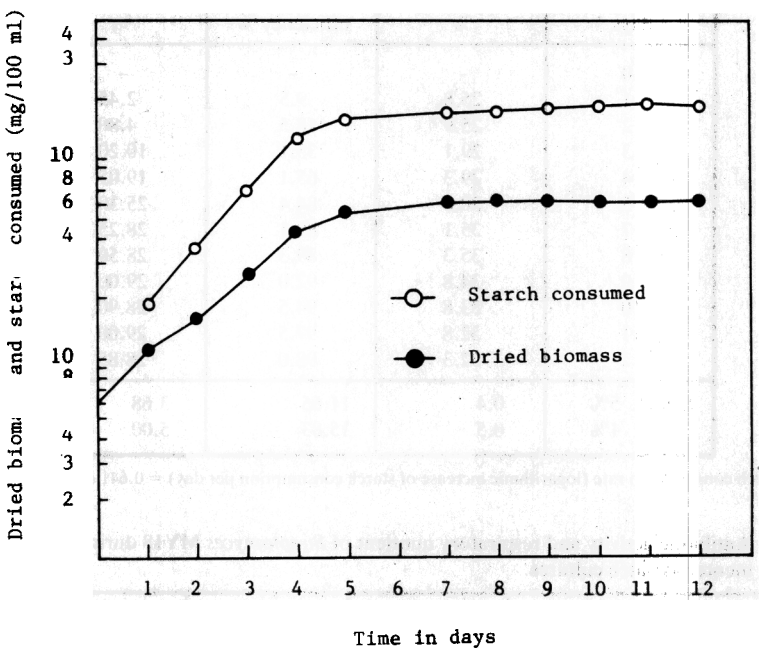
Results and Discussion

Propagation in the Fermentor as Batch Culture

In this experiment, *S. aureofaciens* MY18, which showed high efficiency in the antibiotic activity, was grown in fermentor as batch culture to study its growth, growth parameters and antibiotic activity under a constant environment. The working volume of fermentor was one litre. The aeration and pH were controlled at 600 ml/min (flow rate of air) and 7.0, respectively.

Figure 1 and Table 1 show the growth density as indicated by dry wt of biomass, starch consumed, yield factor (amount of biomass per 100 grams of starch consumed), starch utilization efficiency (amount of consumed starch per 100 grams of original starch) and effective yield (amount of biomass per 100 grams of original starch). It is clear from these results that *Streptomyces* MY18 grew rapidly during the

first 4 days of incubation (exponential phase) with 0.4987 day^{-1} specific growth rate, 1.3899 day doubling time and 2.8777 number of doubling times. Thereafter, the growth rate of this strain decreased gradually (phase of decelerating growth) followed by a constant density of growth (stationary phase) up to the end of incubation period. Starch consumed during the incubation time also exhibited the same trend of growth showing 0.641 day^{-1} specific starch consumption rate. The efficiency of the organism to convert start into biomass (yield factor) ranged from 25.8 to 35.3%. This means that this strain utilizes 100 mg starch to form 25.8 to 35.3 mg of dry biomass.



Growth curve of *S. aureofaciens* MY 18 and starch consumed during propagation in fermenter as a batch culture.

Specific growth rate	=	0.4987 day
Doubling time	=	1.3899 day
Number of doubling times	=	2.8777

Antibiotic activity, oxygen uptake, carbon dioxide production, and respiratory quotient of *Streptomyces* MY18 are shown in Table 2. Results indicate that the antibiotic activity increased gradually with the increase of incubation time reaching its maximum value (40 mm inhibition zone) on the 9*th* day of incubation. This value was significantly higher when compared to inhibition zones of the first 8 days of incubation. When optical density of culture filtrate [The O.D. of golden yellow pigment of the culture can be used as an indication of antibiotic activity for strain under investigation[17]] was determined, it exhibited the same trend of inhibition zone where the

peak (0.51) was recorded on 9*th* day of incubation. Although the oxygen uptake and carbon dioxide production increased gradually during the first 7 days of incubation followed by a gradual decrease up to the end of incubation period, the respiratory quotient remained stable up to the fifth day.

TABLE 1. Yield factor, starch utilization efficiency and effective yield during the propagation of *Streptomyces* MY 18 in fermenter as batch culture.

Time in days	Yield factor %	Starch utilization efficiency %	Effective yield %
0	–	–	–
1	25.8	9.5	2.45
2	25.9	18.5	4.80
3	29.1	35.1	10.20
4	29.3	65.1	19.05
5	30.0	84.4	25.30
7	35.1	89.1	28.25
8	35.3	89.3	28.50
9	34.8	92.0	29.00
10	33.8	94.5	28.90
11	32.8	97.5	29.00
12	32.3	98.0	28.85
L.S.D. 5%	0.4	11.65	3.68
1%	0.5	15.83	5.00

Specific starch consumption rate (logarithmic increase of starch consumption per day) = 0.641 day

TABLE 2. Antibiotic activity and respiratory quotient of *Streptomyces* MY18 during propagation in fermenter as batch cultures.

Time in days	Inhibition zone (mm)	O.D. of culture color	Oxygen m mole / l culture	CO_2 m mole / l culture	RQ*
0	–	–	–	–	–
1	14	0.08	0.023	0.024	1.04
2	16	0.15	0.038	0.039	1.03
3	19	0.25	0.059	0.064	1.08
4	25	0.37	0.101	0.105	1.04
5	29	0.40	0.165	0.171	1.03
7	30	0.41	0.212	0.279	1.32
8	35	0.45	0.145	0.178	1.23
9	40	0.51	0.080	0.095	1.19
10	39	0.50	0.060	0.071	1.18
11	38	0.48	0.050	0.065	1.30
L.S.D. 5%	2.66	0.04	0.017	0.021	0.031
1%	3.55	0.06	0.023	0.029	0.042

Test organism : *Staphylococcus aureus*
*RQ = Respiratory Quotient.

Comparing the growth density and antibiotic activity of *Streptomyces* MY18 with those observed in shaken culture[17], it could be stated that the amount of growth, specific growth rate, and antibiotic activity were higher in the fermentor than in shaken cultures. This could be due to the maintenance of the cultures in the fermentor under controlled conditions.

Continuous Cultures

Tables 3 and 4 show the biological activity of *S. aureofaciens* MY18 during its propagation in the fermentor as continuous cultures at different flow rates (amount of fresh medium input/h): 6.35, 10.00, 19.29, 25.50 and 32.10 ml/h. Results indicate that four steady states were obtained from the tested dilution rates where all the biological activities tested were more constant. The growth density of *Streptomyces* MY18 increased gradually with the increase of dilution rate being 323.2, 413.3, 604.5 and 734.3 mg/100 ml (the mean of each steady state) for 0.00635, 0.01, 0.01929 and 0.0255 h^{-1} dilution rates, respectively. At a dilution rate of 0.0321 h^{-1}, the biomass decreased gradually during the six days of propagation. It means that supplying of the culture by this dilution rate led to a washing out phenomenon. Thus, the optimum dilution rate for growth was 0.0255 h^{-1}.

TABLE 3. Biological activity of *Streptomyces* MY 18 during propagation in fermenter as continuous cultures at different dilution rates.

Dilution rate (h^{-1})	Growth (dry wt)	Starch		Crude antibiotic	Inhibition zone (mm)	Oxygen m mole/l culture	CO_2 m mole/l culture	RQ
	mg/100 ml	Input mg/h	Consumed mg/h	mg/100 ml				
0.00635	323.2	127	126	43.2	21.2	0.129	0.147	1.140
0.01000	413.3	200	194	54.4	25.2	0.151	0.182	1.210
0.01929	604.5	385	381	64.7	29.5	0.183	0.222	1.213
0.0255	734.3	510	498	72.5	33.8	0.213	0.277	1.300

TABLE 4. Biological activity of *Streptomyces* MY 18 during propagation in fermenter as continuous cultures at 32.10 ml/h flow rate (D = 0.03210 h^{-1}).

Time in days	Growth (dry wt)	Starch		Crude antibiotic	Inhibition zone (mm)	Oxygen m mole/l culture	CO_2 m mole/l culture	RQ
	mg/100 ml	Input mg/h	Consumed mg/h	mg/100 ml				
0	730	642	627.4	71.2	35	0.213	0.274	1.286
1	524	642	452.4	50.4	24	0.189	0.201	1.063
2	332	642	284.4	30.5	17	0.165	0.169	1.024
3	210	642	181.5	22.3	12	0.142	0.145	1.021
4	132	642	113.9	12.4	11	0.118	0.121	1.025
5	84	642	69.1	7.5	0	0.094	0.101	1.074
LSD 5%	87.3	-	75.3	8.5	4.2	0.016	0.022	0.036
1%	118.4	-	102.1	11.6	5.7	0.021	0.030	0.049

Growth outlet, starch consumed, oxygen uptake, CO^2 production and respiratory quotient exhibited the same trend of growth. These parameters reached their peak at the steady state of 0.0255 h^{-1} dilution rate where their values were 187.27 mg/h, 498 mg/h, 0.213 m mole/h/1, 0.277 m mole/h/1, and 1.30, respectively. Except the value of respiratory quotient, these figures are significantly higher at this dilution rate compared to other rates.

With respect to antibiotic activity at different steady states, it was found that this activity gave a constant value at each steady state and this value increased gradually with the increase of dilution rate reaching its maximum value at 0.0255 dilution rate, thereafter no steady state was recorded (washing out). The maximum amount of crude antibiotic was 72.52 mg/100 ml culture (productivity per day = 443.8 mg) at the steady state of 0.255 h^{-1} dilution rate.

In this investigation, the specific growth rate equals dilution rate at each steady state. So the maximum specific growth rate for *Streptomyces* MY18 was 0.0255 h^{-1} (0.612 day^{-1}). This value of specific growth rate was higher than that observed in the fermentor as a batch culture (0.4987 day^{-1}).

In general, continuous cultivation of *Streptomyces* MY18 gave higher biological activities than batch cultures. The highest productivity of antibiotic by this method of cultivation may be due to the propagation of organism at a steady state where the addition of nutrients, consumption rate of starch, uptake of oxygen, and production of carbon dioxide were constant and in their highest efficiency, in addition to the continuous removing of metabolic products. The low growth and yield factor in batch cultures may be due to the accumulation of waste products of metabolism and/or the depletion of nutrients in the medium, while in the continuous cultures, metabolic waste products were removed, at the same time, fresh medium could be added to the culture[2,18,19].

The maximum dilution rate (0.0255 h^{-1}) obtained from the continuous culture for this organism also reveals that the highest efficiency in the utilization of nutrients was occurred, and the increase of dilution rate than 0.255 h^{-1} did not give it the opportunity to utilize all of the nutrients added. The excess of these nutrients was washed out with the culture outlet causing washing phenomenon. This is clear in the last dilution rate (0.0321h^{-1}) where no steady state was observed, the biomass and antibiotic in the fermentor gradually decreased. It means that the last dilution rate (feeding rate) was higher than the specific growth rate, and was not suitable for propagation of the test organism and also for the production of antibiotics.

Effect of pH on Streptomyces MY18 in Continuous Cultures

During the propagation of *Streptomyces* MY18 in the fermentor as continuous cultures at 0.0255 h^{-1} dilution rate (the maximum dilution rate as described before), different pH values were used to study the respiration rate of the organism. The pH values were achieved by addition of HCl (1*N*) or NaOH (1*N*) automatically by the pH controller. The following pH values were applied: 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11.

Results in Table 5 showed that the highest amount of oxygen uptake and carbon

dioxide formation were detected at pH 7 where their values were 0.224 ad 0.280 m mole/1 culture (RQ = 1.25). The increase or decrease of pH from this level decreased the amount of oxygen uptake and CO_2 formation sharply.

TABLE 5. Effect of different pH values of Oxygen U and carbon dioxide formation by *streptomyces* MY18 as continuous cultures at 25.5 flow rate (D = 0.0255 h^{-1}).

pH values	Oxygen m mole / l culture	CO_2 m mole / l culture	RQ
2	0.004	0.005	1.2
3	0.005	0.005	1.0
4	0.007	0.006	0.8
5	0.005	0.006	1.2
6	0.092	0.095	1.0
7	0.224	0.280	1.2
8	0.118	0.166	1.4
9	0.033	0.035	1.0
10	0.018	0.020	1.1
11	0.018	0.022	1.2
L.S.D. 5%	0.020	0.025	0.0
1%	0.026	0.034	0.0

It is clear from these results that the optimum pH for propagation of *Streptomyces* MY18 is 7.0. This result is in line with that observed by many investigators[20-22], who reported that the suitable pH value for propagation of *Streptomyces aureofaciens* ranged from 6.8 to 7.5. On the contrary, it was found that the optimal pH for *Streptomyces aureofaciens* ranged from 5.8 to 6.0[23]. This variation may be due to the bacterial strain used.

Activity of Extracted Crude Antibiotic

In this investigation, the antibiotic extracted from the cultures of *Streptomyces* MY18 by *n*-butanol, butanol followed by distilled water or butanol followed by acetone was tested for antibiotic activity. Results in Table 6 indicated that the

TABLE 6. Amount of crude antibiotics produced by *Streptomyces* MY 18 (extracted with different solvents).

Extraction by	Crude antibiotics (mg / l culture)
Butanol only	695.1
Butanol followed by distilled water	237.3
Butanol followed by acetone	345.2
L.S.D. 5%	113.79
1%	159.55

amount of extracted crude antibiotic varied from one solvent system to another. When these fractions were tested for antibiotic activity at different concentration (Table 7), it was found that all fractions exhibited antibiotic activity. A slight high antibiotic activity was recorded in the crude antibiotic extracted by water. On the other hand, the increase of antibiotic concentrations led to an increase in the inhibition zone among all fractions. Results in Table 8 show that the increase of tetracycline concentration led to an increase in the diameter of inhibition zone.

TABLE 7. Antibiotic activity of crude antibiotics of *Streptomyces* MY18.

Crude antibiotic Streptomyces MY 18 µg/ml	Inhibition zone* (mm)		
	a	b	c
10	11	12	11
20	13	14	12
30	15	17	14
40	19	20	18
50	22	24	21
60	27	29	26
80	39	41	37
90	40	45	40
L.S.D. 5%	4.7	5.0	4.6
1%	6.4	7.0	6.3
Correlation coefficient	0.9856	0.9873	0.9752

a – Extraction by *n*-butanol
b – Extraction by *n*-butanol followed by dist. water
c – Extraction by *n*-butanol followed by acetone
*Test organism: *Staphylococcus aureus*

TABLE 8. Antibiotic activity of tetracycline.

Tetracycline mg/ml	Inhibition zone* (mm)
10	12
20	14
30	19
40	23
50	29
60	32
70	36
80	43
90	48

*Test organism	*Staphylococcus aureus*
Regression analysis	Y = 0.457 X + 5.61
Correlation coefficient (R)	= 0.9957

Comparing antibiotic activity of the crude antibiotic extracted with tetracycline, it could be stated that the extracted antibiotic exhibited approximately a similar activity to the tetracycline.

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إكثـــار ستربتـوميســس أوريـوفاشينس أم. واي. ١٨ في الـمُخمِّر كمـزارع ذات الدفعـــة الواحـــدة والمستـمرة

فهد عبد الرحمن الفاسي
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جـــدة ، المملكة العربية السعودية

المستخلص . تمت تنمية ستربتـوميسس أوريوفـاشينس أم. واي. ١٨ (عزلة محلية) في المُخَمِّر كمزارع ذات دفعة واحدة ومستمرة (كيموستات) . وقد أدت التنمية تحت ظروف ثابتـة في مزارع الدفعة الواحدة إلى زيادة إنتاجية الكتلة الحيوية والمضاد الحيوي . وكان معـامل التنفس ثابتًا ، في حين زاد كلٌّ من الأوكسجين المستهلك وثاني أكسيد الكربون الناتج تدريجًا ليصلاً إلى أقصاهما في اليوم السابع من التحضين .

أوضحت التنمية المستمرة عند معدلات تخفيف مختلفة أن أعلى كفاية في الاستفادة من المواد الغذائية المضافة قد تحققت عند معدل تخفيف ٠,٠٢٥٥ / ساعة (٢٥,٥ مل/ ساعة معدل إضافة) . وقد أعطت حالة ثبات هذا المعدل من التخفيف قِيمًا ثابتة لكلٍّ من النشا المستهلك ، وكفاية الاستفادة من النشا ، والأوكسجين المستهلك ، وثاني أكسيد الكربون الناتج . وقد أدت زيادة معدل التخفيف إلى ٠,٠٣٣١ / ساعة (٣٢,١ مل/ ساعة) إلى حدوث انخفاض تدريجي في كل العينات الحيوية مسببة ظاهرة الإزاحة . لوحظ أن درجة الأس الهيدروجيني المُثلى في مزرعة الكيموستات هي ٧ ، وأن زيادتها أو انخفاضها عن هذا المستوى قد أدى إلى انخفاض معدل التنفس ، وبالتالي انخفاض إنتاج الكتلة الحيوية والمضاد الحيوي . وقد أظهر المضاد الحيوي الخام المستخلص بالبيوتانول نشاطًا ملموسًا مقارنًا بالتتراسيكلين .