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Kinetic Studies of Gluconic Acid Fermentation in Horizontal Rotary Fermenter by *Pseudomonas ovalis*

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Abstract

Characteristic kinetics of gluconic acid fermentation by *Pseudomonas ovalis* B 1486 have been studied in a horizontal rotary fermenter (HRF). Effects of physical parameters like nutrients, pH, temperature and volumetric oxygen transfer coefficient on the yield of gluconic acid are examined. Values of kinetic constants have been obtained from graphical analysis and these data are used in computer simulation by the Runge-Kutta method in an ICL 1909 computer. Representative kinetic equations derived from the best fit are proposed. Results of the analysis are described in detail.

Introduction

Microbial production of gluconic acid is one of the oldest processes used in the past in horizontal rotary fermenter (HRF).[1] In extreme gas-liquid interaction this system has been studied in HRF as a suitable reactor.[2] Apparent dependence of microbial activity on equipment type has been reported by Aiba and Shoda.[3] It has been suggested that many of the problems of sparged stirred fermenters can be resolved in HRF.[4] The system is significantly important on account of a number of outstanding advantages, namely excellent oxygen dispersion, efficient removal of metabolic products by individual cells and availability of fresh nutrients to the active cells.[5] Despite these important findings and other advantages like low power consumption per unit volume, simultaneous oxygen uptake from dissolved and gas phases and easier solution of scale up problems of HRF, engineering data available so far are scanty. The present studies provide data on the effect of control variables (nutrient concentration, pH, temperature and k_La) on state variables like cell growth and product yield and kinetic characteristics of gluconic acid fermentation in HRF. These data studied in conjunction with those obtained by earlier workers make it possible to analyse the system more precisely.

Materials and Methods

Pseudomonas ovalis B 1486[6] was used in the studies. Fermentations were conducted in the same synthetic medium used by those authors. The fermenter was a standard glass vessel (QVF) with a bulk capacity of 2.5 *l*, supported on a central shaft on either side and rotated by D.C. motor (1420 rpm, 0.25 HP) through belt drive. The speed of rotation could be controlled between zero and 150 rpm. A schematic diagram of the apparatus is shown in Fig. 1. D.O. probe (Universal Oxygen Analyser Model RA, Oriental Electric Co. Japan) monitored point concentrations with the progress of fermentation. Ingold steam sterilizable pH electrodes were used with controller and recorder (Analytical Measurements Ltd. Surrey MOC$_2$). K_La values were measured by oxygen balance technique.[7] The percentage oxygen in gas phase was measured by paramagnetic oxygen analyser (DCL Servomax Oxygen Analyser Type 83; Servomax Control Ltd. Crowborough).

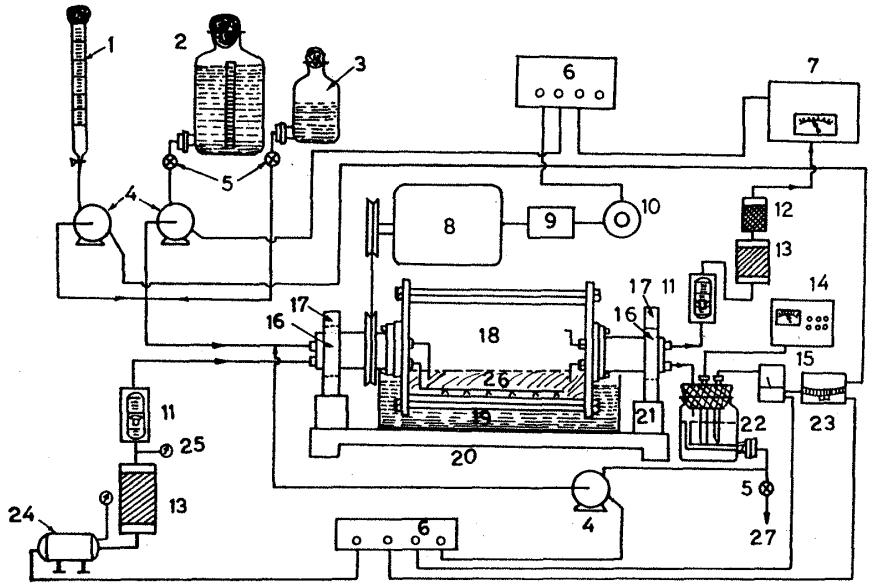

Fig. 1. Schematic diagram of the experimental set up.
1. Alkali reservoir 2. Medium reservoir 3. Inoculum vessel 4. Micropump 5. Valve 6. Switchboard 7. Gas phase oxygen analyser 8. Motor 9. Rectifier 10. Speed regulator 11. Sterile rotameter 12. Moisture absorber 13. Air filter 14. D.O. recorder 15. pH meter 16. Ball bearing 17. Pumner block 18. HRF 19. Controlled temperature water bath 20. Platform 21. Movable arm 22. Sample bottle 23. pH controller 24. Air compressor 25. Pressure guage 26. Air sparger 27. Sample point

Oxygen transfer values were controlled by regulating rotation speed of HRF. Temperature of fermentation was controlled by keeping the fermenter partially immersed in a water bath whose temperature was kept precisely at the desired level by a combined warm and chilled water recirculation system under thermostatic control. Samples drawn from sampling bottle at regular time intervals were analysed for cell, glucose, ammonium acetate, gluconolactone and gluconic acid concentrations. Cell concentration was measured in terms of ultimate optical density (C_x, UOD ml^{-1}) in SP Pye 600 spectrophotometer at 600 nm using the empirical relation

$$C_x = n' OD_a - OD_b$$

where

$$OD_a = OD_m \exp 0.615\ (OD_m - 0.12)$$

to compensate the deviation from Lambert Beer's law which is valid in the OD range 0–0.12 in the equipment. Concentration of glucose was measured by DNS method,[8] ammonium acetate in terms of ammonia by Nesseler's spectrophotometric method[9] and gluconolactone and gluconic acid by Lien's method.[10]

Result

Effect of components of synthetic medium Submerged fermentations were conducted for 14 hr in HRF (working volume 0.9 l) at pH 6.8, temperature 29°C, aeration rate 0.5 vvm (27 l hr^{-1}) at a pressure of 3 atm and rotation speed of 120 rpm. k_La was maintained at 187 hr^{-1}. The synthetic medium contained all nutrients except the one whose effect was to be studied. Results are given in Table 1. These indicate that in the synthetic medium used, ammonium acetate might be limiting nutrient for the growth of *P. ovalis* B 1486.

Growth limiting nutrient Two sets of fermentations were conducted under the above pH, temperature and aeration conditions. In the first set, media contained all nutrients with varying ammonium acetate concentrations. In the second, the effect of

Table 1. Effect of components of the synthetic medium on cell growth and gluconic acid yield in HRF.

Fermentation time hr	Growth of medium					Gluconic acid production on medium				
	With all nutrients (UOD/ml)	Devoid of $MgSO_4 \cdot 7H_2O$ (UOD/ml)	Devoid of KH_2PO_4 (UOD/ml)	Devoid of ammonium acetate (UOD/ml)	Devoid of glucose (UOD/ml)	With all nutrients (UOD/ml)	Devoid of $MgSO_4 \cdot 7H_2O$ (mg/ml)	Devoid of KH_2PO_4 (mg/ml)	Devoid of ammonium acetate (mg/ml)	Devoid of glucose (mg/ml)
0	0.10	0.10	0.08	0.08	0.08	—	—	—	—	—
1	0.11	0.10	0.08	No growth	No growth	0.12	—	—	No yield	No yield
2	0.169	0.12	0.10	//	//	0.60	0.08	0.28	//	//
3	0.215	0.14	0.12	//	//	1.08	0.22	0.52	//	//
4	0.315	0.16	0.16	//	//	1.92	0.33	0.93	0.80	//
5	0.480	0.20	0.22	//	//	3.27	0.62	2.12	—	//
6	0.873	0.34	0.35	//	//	5.68	1.28	4.25	1.50	//
7	1.25	0.48	0.42	//	//	9.72	—	5.38	—	//
8	1.732	0.56	0.48	//	//	15.08	3.35	—	2.00	//
9	2.203	0.62	0.52	//	//	22.10	—	11.25	—	//
10	2.20	0.62	0.52	//	//	30.86	8.48	—	—	//
11	2.20	0.62	0.52	//	//	37.00	—	17.33	—	//
12	2.20	—	—	//	//	42.14	15.49	—	2.08	//
13	—	—	—	//	//	45.68	—	23.28	—	//
14	—	—	—	//	//	46.82	20.25	25.11	3.00	//

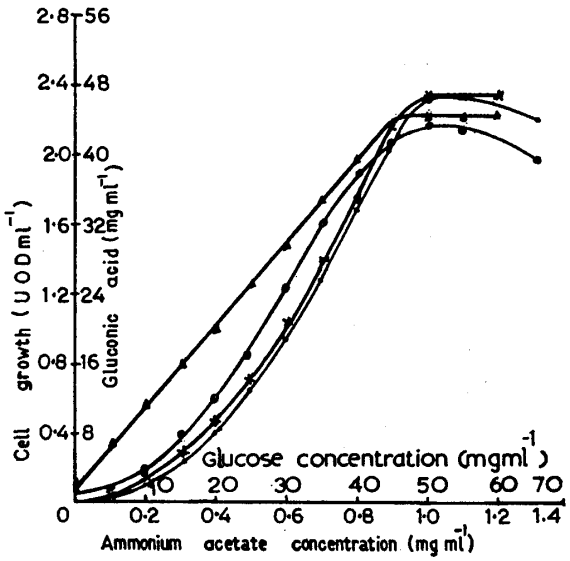

Fig. 2. Effect of ammonium acetate and glucose on cell growth and gluconic acid yield.

different glucose concentrations was observed. Results are given in Fig. 2. Increasing the concentration of ammonium acetate above 1.0 mg/ml resulted in neither decrease nor increase of growth or of gluconic acid yield. Up to this level of ammonium acetate, cell concentration increased linearly. For glucose, optimum growth and acid yield were obtained at 51.0 mg/ml. Unlike ammonium acetate, increasing concentration of glucose above 51.0 mg/ml had detrimental effects on both the state variables. No inhibition of growth or gluconic acid yield could be observed by adding different concentrations of sodium gluconate to the medium. Also, there was no linear increase of growth with increase of glucose concentration in the medium. Cells grown on two glucose concentrations (51.0 mg/ml and 90.0 mg/ml) were separated by centrifugation and inactivated by holding in an aerosol of a saturated disinfectant (Dettol, Reckitt and Colman India Ltd.) for 4 hr. Cells were then dried at 60°C for 3 hr and the number per unit dry mass was taken for each medium. In the former medium this was 5.0×10^{13} and in the latter 1.2×10^{13} per mg dry cells. Specific glucose uptake rate in former case was 5.5 mg UOD^{-1} hr^{-1} and in the latter 3.0 mg UOD^{-1} hr^{-1}. Average surface area of unit mass of dried cells measured by methylene blue dye adsorption technique[11] was 3.0 $(\mu m)^2$ in the former medium and 0.97 $(\mu m)^2$ in the latter, showing an inhibition due to plasmolytic effect. These results showed, therefore, that ammonium acetate is limiting nutrient for the growth of this organism.

Responses to pH, temperature and k_La Three sets of experiments were conducted at different pH, temperature and k_La values. In each set three independent runs were performed. Since results were consistent average values were taken; these are given in Fig. 3. Maximum cell growth and specific gluconic acid yield were obtained at pH 6.8 (in the range studied) and 29°C. Residual sugar (RS) level at pH values <6.8 is significant. Specific gluconolactone yield, however, was optimum at pH 5.8. At temperature lower and higher than 29°C values of both the state variables declined. With k_La in the liquid lower than 150 hr^{-1} these yields were reduced, but higher values did not produce any change.

Batch kinetics Fermentation conducted at 29°C, pH 6.8 and k_La 187 hr^{-1} with initial cell concentration of 0.10 UOD ml^{-1} produced the kinetic data shown in Fig. 4. At the end of the run cell concentration on dry weight basis was 2.8 mg ml^{-1} corresponding to spectrophotometric value of 2.2 UOD ml^{-1}. The linear relation between specific growth rate and specific gluconic acid production rate (Fig. 5) calculated from the data in Fig. 4

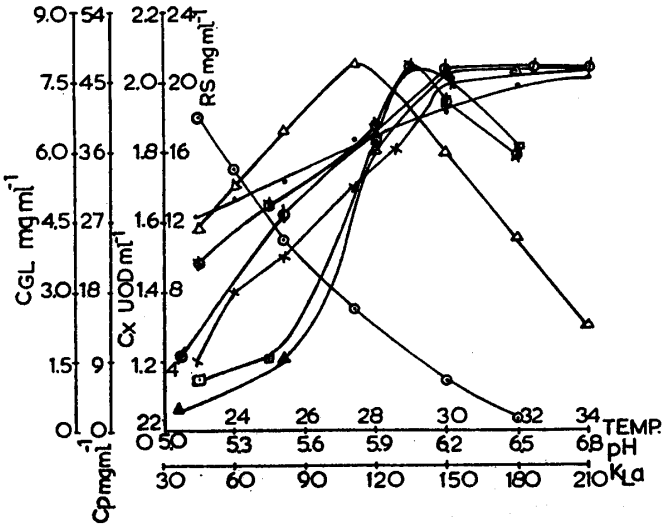

Fig. 3. Responses to pH, temperature and k_La.

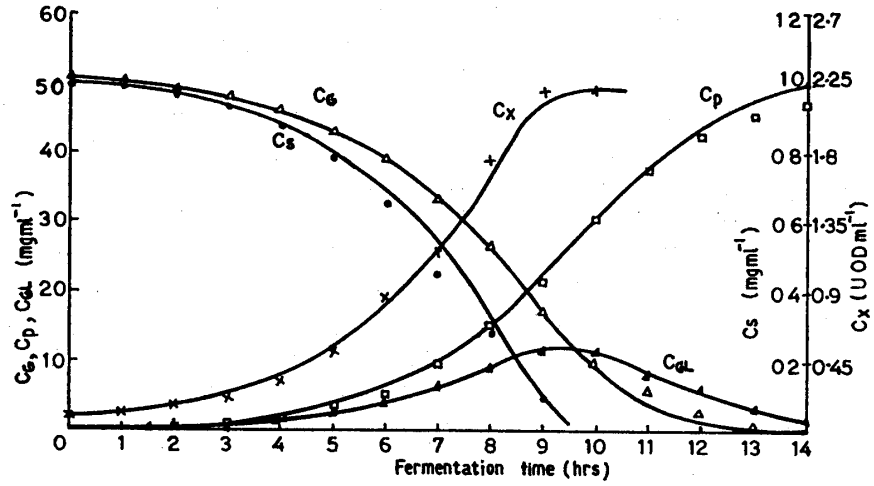

Fig. 4. Fitting of experimental data into computed results.

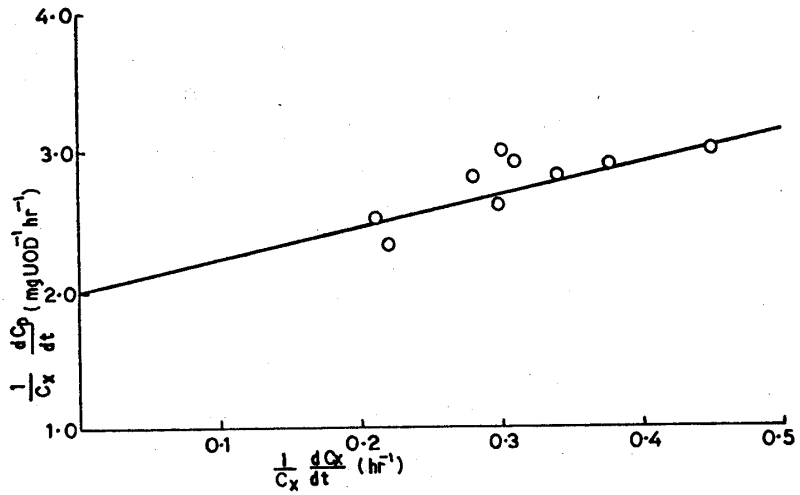

Fig. 5. Relation between specific growth rate and specific gluconic acid production rate.

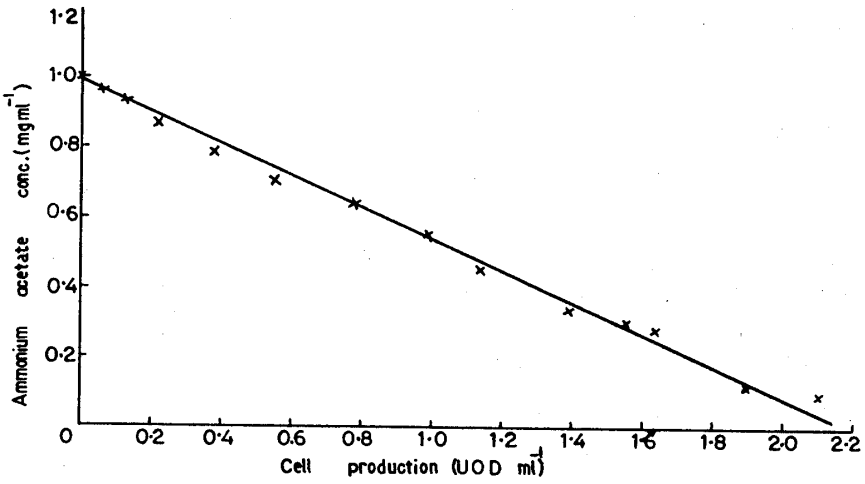

Fig. 6. Relation between limiting nutrient consumption and cell growth.

illustrated the growth-associated nature of this fermentation.

Growth rate and limiting nutrient Ammonium acetate being the limiting nutrient, the rate of change of its concentration (C_S) should be proportional to the rate of change of cell growth (C_X) of *P. ovalis*. Thus

$$dC_S/dt=(-1/Y_S)\ (dC_X/dt) \tag{1}$$

Its solution under initial conditions

$$C_S=C_{S_0},\ C_X=C_{X_0} \quad \text{at} \quad t=0$$

is

$$C_S=C_{S_0}-(1/Y_S)\ (C_X-C_{X_0}) \tag{2}$$

This linearity was obtained from the actual plot (Fig. 6) of C_S *vs* $(C_X-C_{X_0})$ from the data in Fig. 4 generating a yield coefficient of 2.25 UOD mg^{-1}. Represented in the form of the double reciprocal of specific growth rate *vs* limiting nutrient concentration (Fig. 7), namely,

$$C_X/(dC_X/dt)=(K_s/\mu_m)\ (1/C_S)+(1/\mu_m) \tag{3}$$

this concurs with Monod's model. Values of μ_m and K_S were obtained from the plot as

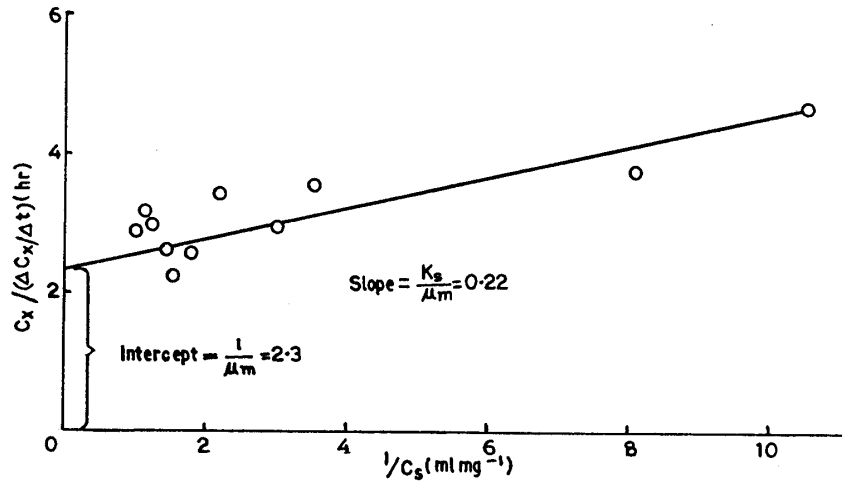

Fig. 7. Double reciprocal relation between limiting nutrient concentration and specific growth rate.

0.43 hr^{-1} and 0.095 mg ml^{-1} respectively. These were further checked with theoretically calculated growth time

$$t=(\beta/\alpha)\ \ln\left(\frac{\alpha-1}{\alpha-N}\right)+\nu\ \ln N \tag{4}$$

and were also compared with actual growth time (Fig. 8). Equation (4) was obtained by substituting Eq. (2) in Monod's model with the necessary rearrangements. Deviation observed (Fig. 8) was checked by χ^2 (chi-square) test which showed a high degree of confidence for the results between 1.5 hr to 9 hr of growth.

Gluconic acid production Reaction scheme of gluconic and production may be represented by the following equations[12,13]

$$C_5H_{11}O_5CHO+H_2O \rightleftarrows C_5H_{11}O_5CH(OH)_2 \tag{5}$$

$$C_5H_{11}O_5CH(OH)_2+NAD^+ \rightleftarrows C_6H_{10}O_6+NADH+H^++H_2O \tag{6}$$

$$NADH+H^++2\ Cyt\ C(Fe^{+3}) \rightleftarrows NAD^++2\ Cyt\ C(Fe^{+2})+2H^+ \tag{7}$$

$$2\ Cyt\ C(Fe^{+2})+2\ Cyt\ a_3(Fe^{+3}) \rightleftarrows 2\ Cyt\ C(Fe^{+3})+2\ Cyt\ a_3(Fe^{+2}) \tag{8}$$

$$2\ Cyt\ a_3(Fe^{+2})+1/2\ O_2+2H^+ \rightleftarrows 2\ Cyt\ C\ a_3(Fe^{+3})+H_2O \tag{9}$$

$$C_6H_{10}O_6+H_2O \longrightarrow C_6H_{12}O_7 \tag{10}$$

$$\text{Overall reaction}\quad C_6H_{12}O_6+1/2\ O_2 \longrightarrow C_6H_{12}O_7 \tag{11}$$

Since gluconolactone is accumulated in the broth it is suggested that gluconic acid (C_P) production rate must be proportional to accumulated gluconolactone concentration (C_{GL}). Thus,

$$dC_P/dt=a'C_{GL} \tag{12}$$

where a' is a proportionality constant. Plot of $\Delta C_P/\Delta t$ *vs* C_{GL} is also linear (Fig. 9) having slope a' of 0.775 hr^{-1}.

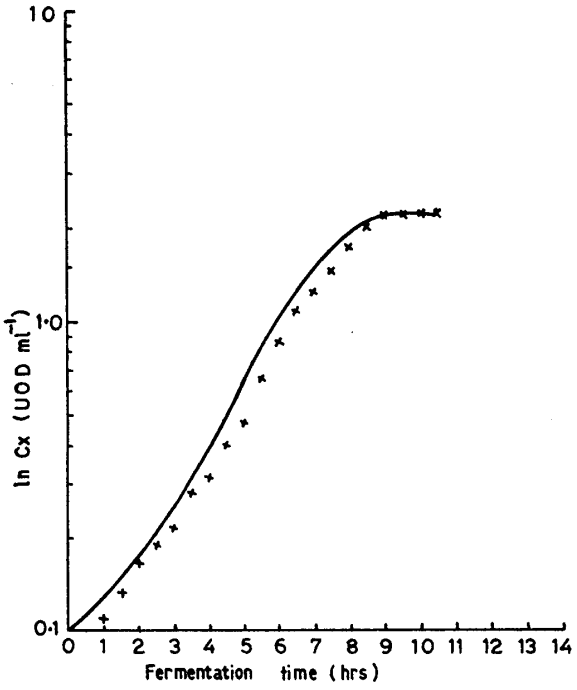

Fig. 8. Calculated and experimental fermentation time of *P. ovalis* B 1486 in HRF.

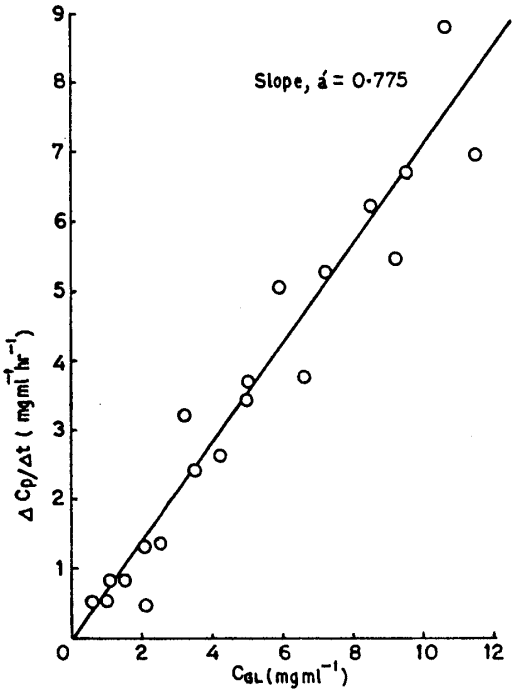

Fig. 9. Relation between gluconolactone accumulation and gluconic acid production rate.

Gluconolactone accumulation Dehydrogenation of glucose (Eq. 5) is followed by hydrolysis through a series of reactions (Eqs. 7–10) to yield gluconic acid. Therefore, the algebraic sum of the rate of gluconolactone accumulation and its hydrolysis should be proportional to the glucose uptake by the cells. Thus

$$dC_{GL}/dt + bC_{GL} = -\alpha'(dC_G/dt) \tag{13}$$

In this equation the constant b takes into account the molecular ratio of glucose to gluconolactone. Its determined value is 0.7 hr^{-1}. A plot (Fig. 10) of the calculated values of $(\Delta C_{GL}/\Delta t)+0.7\ C_{GL}$ against $(\Delta C_G/\Delta t)$ obtained from Fig. 4 showed the validity of Eq. (13). Proportionality constant $\alpha'=1.05$ was obtained from the slope of Fig. 10.

Glucose uptake In cell growth 3 mg/ml of other nutrients are consumed along

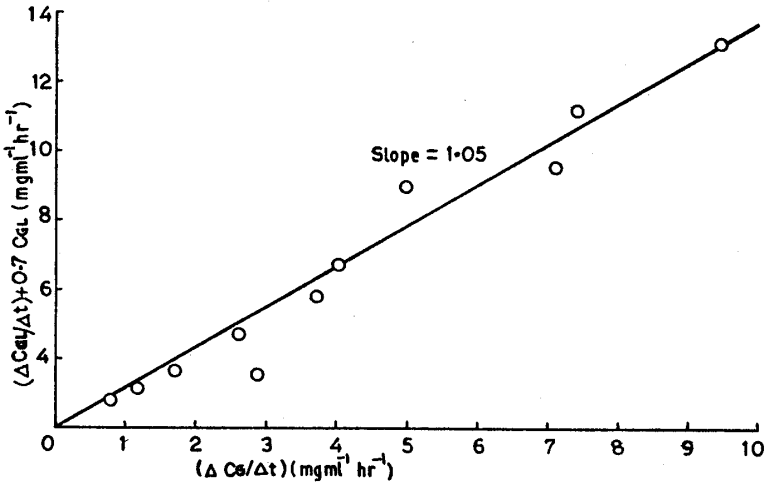

Fig. 10. Plot of algebraic sum of gluconolactone accumulation and hydrolysis rate *vs* glucose uptake rate.

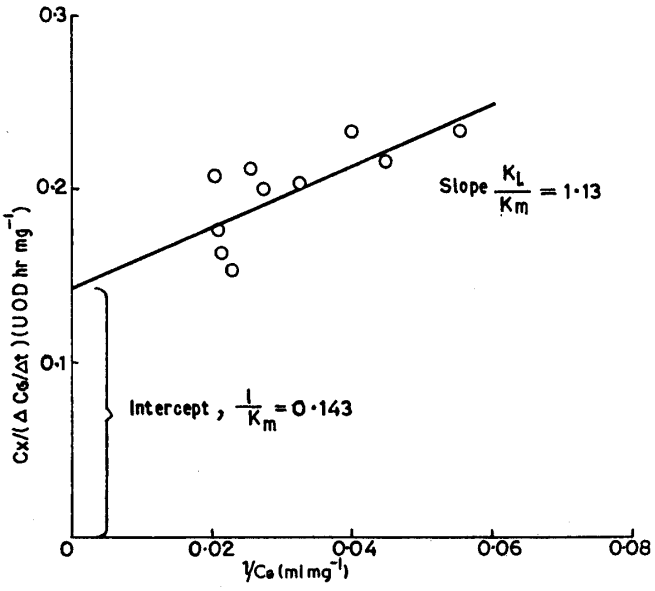

Fig. 11. Double reciprocal relation between glucose concentration and specific glucose uptake rate.

with glucose in producing only 2.8 mg/ml cells. Thus glucose consumption in cell nourishment is very small and can be neglected. Gluconclactone formation is, therefore, essentially dependent on glucose (C_G) uptake, and since the reaction is solely enzymatic[13,14] we can write

$$-dC_G/dt=\left(\frac{K_mC_G}{K_l+C_G}\right)C_X \tag{14}$$

Rearranging in the linear form gives

$$\frac{C_X}{-(dC_G/dt)}=(K_l/K_m)(1/C_G)+(1/K_m) \tag{15}$$

The linearity was illustrated in a plot of $C_X/(\Delta C_G/\Delta t)$ *vs* $1/C_G$ (Fig. 11). Values of K_l and K_m determined from it are 7.91 mg/ml and 7.0 mg UOD^{-1} hr^{-1} respectively. Since the points in Fig. 11 are scattered Eq. (13) and (14) are combined to check the reliability of K_l and K_m values, giving

$$(dC_{GL}/dt)+0.7\,C_{GL}=1.05\{(K_mC_G)/(K_l+C_G)\}(C_X) \tag{16}$$

At high values of C_G (for $t\leqslant 9.0$ hr) the values of $C_G/(K_l+C_G)\simeq 1$ and Eqn. (16) becomes

$$(dC_{GL}/dt)+0.7\,C_{GL}=1.05\,K_mC_X \tag{17}$$

Actual plot (Fig. 12) of Eq. 17 showed a straight line passing through origin (slope=7.35) giving a value for K_m of 7.0 mg UOD^{-1} hr^{-1}. The corresponding value of K_l is determined as 8.0 mg/ml at the ninth hour when C_{GL} shows a maximum value of 11.50 mg/ml. Graphically determined value of K_l is 7.91 which is within 2% of this value. This is taken as 7.91 in this time range and the ratio of K_l/K_m becomes 1.13.

Variation of K_m with time was checked from the plot of $(\Delta C_{GL}/\Delta t)+0.7\,C_{GL}/\{(1.05\,C_GC_X)/(K_l+C_G)\}$ *vs* time (Fig. 13). It showed a constant value of K_m (7.0 mg UOD^{-1} hr^{-1}) upto $t\leqslant 9.0$ hr fermentation. For $t>9.0$ hr there is an abrupt fall of K_m from 7.0 to 5.1 mg UOD^{-1} hr^{-1} in the range 9.0 to 9.5 hr. Beyond this time K_m decreases linearly. This phenomenon will probably be significant and requires further investigation.

Computer simulation Kinetic pattern of gluconic acid fermentation by *P. ovalis* can be represented by the following equations

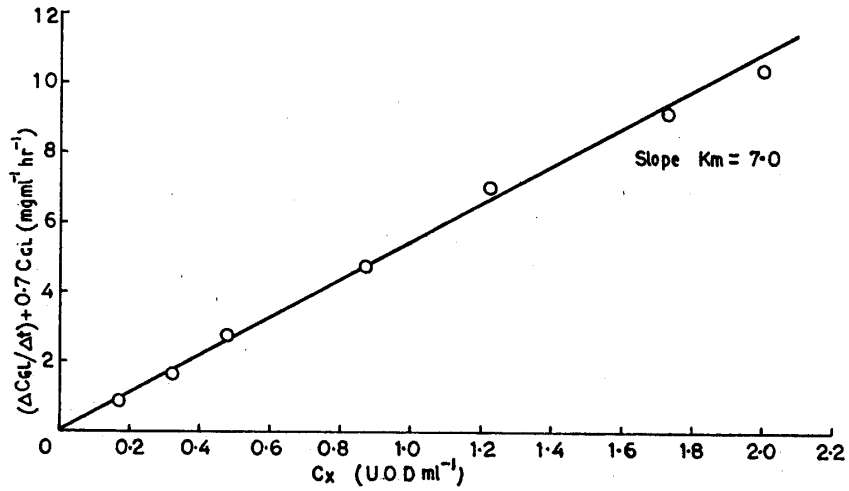

Fig. 12. Relation between cell concentration and algebraic sum of gluconolactone accumulation and its hydrolysis.

$$dC_S/dt = -1/Y_S(dC_X/dt)$$
$$dC_X/dt = \{\mu_m C_S/(K_S+C_S)\}(C_X)$$
$$-dC_G/dt = \{K_m(t)C_G/(K_l+C_G)\}(C_X)$$
$$dC_P/dt = a'C_{GL}$$
$$dC_{GL}/dt = a'(dC_G/dt) - bC_{GL}$$

Numerical solution of these equations was obtained using standard Fourth Order Runge-Kutta Program in a ICL 1909 computer to simulate fermentation kinetics and check the appropriateness of the kinetic equations. The kinetic parameters obtained from graphical analysis were used in simulation. Two linear equations showing variation of K_m with time were obtained as follows;

$$K_m = -3.8\,t + 7.0 \quad (9.0 < t < 9.5 \text{ hr}) \tag{18}$$

and

$$K_m = -1.13\,t + 5.665 \quad (t > 9.5 \text{ hr}) \tag{19}$$

A comparison between the computed and the experimental data is illustrated in Fig. 4. The solid lines represent computed values. A good fit is obtained which allows reasonable confidence in the equations described.

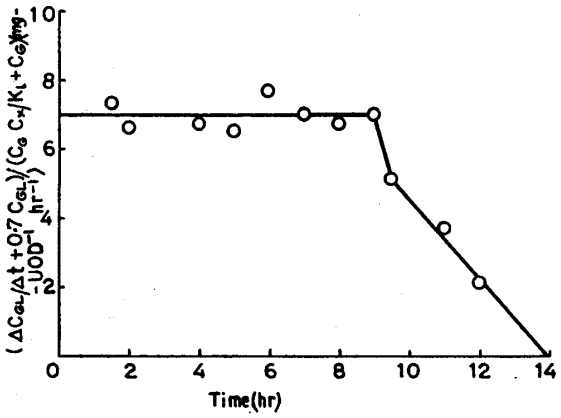

Fig. 13. K_m as a function of time for $t > 9.0$ hr.

Discussion

Experimental results show that the first enzymatic step in gluconic acid production was favoured at pH 5.8 (Fig. 3). At this pH, however, the rate of gluconolactone hydrolysis to gluconic acid is slower which results in accumulation of gluconolactone in large amounts. At pH 6.8 its hydrolysis is faster. Since at pH 6.8 some gluconolactone accumulation is observed its hydrolysis might be controlling. It requires explanation how at higher bulk pH a high rate of gluconolactone formation takes place. Higher glucose concentration than 51.0 mg/ml inhibited both cell growth and gluconic acid yield. Since addition of sodium gluconate to the medium does not produce any inhibition, plasmolytic effect of glucose is observed. Average cell surface reduction at higher glucose concentration shows the presence of such an effect. This in turn causes inhibition. Although gluconolactone accumulation is maximum at pH 5.8, maximum gluconic acid yield resulted at pH 6.8 in the observed range. Also a temperature of 29°C is found to be optimum for gluconic acid yield (Fig. 3). Such results may be interpreted in the light of thin liquid film situation in HRF.

Observed results suggest that the liquid film surface become responsible for the acceleration of gluconolactone formation at higher bulk pH. During rotation of HRF the thin liquid film containing bacterial cells is exposed to air current with the maximum O_2 partial pressure. Many of the cells might be assumed to remain in the gas-liquid interface. All bacteria above the isoelectric point possess negative charge.[13] *P. ovalis* B 1486 is a gram negative bacteria with an isoelectric point around 5.0.[15] Since bulk pH in gluconic acid fermentation is 6.8 *P. ovalis* B 1486 cells possess negative charge as this is above the isoelectric point. When such cells are at gas-liquid interface, the gas phase acts as an insulator and large potential gradient is set up in the liquid film. The net effect is to attract H^+ ions to the interface, thus lowering the pH on the cell surface in the liquid film. Quantitatively, this change may be represented[16] by

$$C_{Hs}^{+}=C_{Hb}^{+}+\cdot e^{-(\epsilon\phi/KT)}$$

or

$$pH_S=pH_b+\varepsilon\psi/KT \tag{20}$$

Since the net charge on the bacterial cell is negative the pH in the surface phase in the liquid film will be lower than in the bulk liquid phase. This effect is enhanced with the increase in the number of cells in the interface. It appears therefore that due to attraction of H^+ ions to the cell surface in the thin liquid film, the pH in the vicinity of the cell surface decreases to the level favourable for gluconolactone formation. The pH on the bacterial surface in the liquid being lower than that in the bulk liquid, lactone formation is accelerated. Accumulation of gluconolactone in the bulk suggests that its hydrolysis is the controlling factor in the greater gluconic acid production. It is most likely that increase in lactone production rate occurs in the liquid film. As soon as the liguid film progressively mixes with the bulk it contributes to rising of lactone concentration in the bulk liquid. At the same time the pH of the liquid film returns to the higher bulk pH at which lactone hydrolysis is higher. The net effect results in an increase in the rate of gluconic acid production at pH 6.8.

Temperature effect (Fig. 3) shows that below 29°C cell growth is less. Negative charge distribution on the cell surface may, therefore, be assumed low. As a result net attraction of H^+ ions on the cell surface in the film is decreased and the cell surface pH does not come down to the required level favourable for gluconolactone formation. This causes lower gluconic acid yield in the bulk. On the other hand, temperatures greater than 29°C decrease cell surface pH to the level lower than that required for optimum gluconolactone

formation. Such a decrease of pH in the liquid with increase in temperature has been reported in literature.[15] Theoretically this decrease can be shown from Eq. 20. By proper rearrangement it can be written as

$$(pH_{S_1}-pH_b)/(pH_{S_2}-pH_b)=T_2/T_1 \quad (21)$$

Since pH_b is maintained constant during fermentation Eq. (21) indicates that cell surface pH varies inversely with temperature. So it is likely that temperatures above 29°C cause the surface pH to drop below the required level thereby lowering the formation of gluconolactone at the cell surface. The net effect being exhibited showing less gluconic acid yield in the broth.

Predicted kinetic equations from graphical analysis of the experimental data could be simulated by computational analysis using ICL 1909. Growth-associated fermentation kinetics of similar characteristics may be applied for computer simulation of such a system.

Conclusion

Gluconic acid fermentation by *P. ovalis* B1486 appears to be a growth-associated process. Ammonium acetate acts as a limiting nutrient for the growth of this organism. In HRF, a thin cell-containing film around the peripheral surface of the vessel containing cells acids to the increase of gluconic acid fermentation at higher pH by enhancing gluconolactone formation on the cell surface by an interfacial mechanism. In a microbial system this mechanism is possibly optimum at a particular temperature. For *P. ovalis* B 1486 system this occurs at 29°C. Based on a fairly good match between experimental and computed results it is concluded that the kinetic equations obtained from batch data are appropriate.

Nomenclature

a' =proportionality constant or hydrolysis constant
b =a constant
C_G =glucose concentration, mg/ml
C_{GL} =gluconolactone concentration, mg/ml
C_P =gluconic acid concentration, mg/ml
C_S =ammonium acetate concentration, mg/ml
C_{S_0} =ammonium acetate concentration at $t=0$
C_X =cell concentration, UOD/ml
C_{X_0} =Cell concentration at $t=0$
K =Boltzman constant
K_l =Michaelis constant for lactone formation, mg/ml
K_m =Velocity constant for lactone formation, mg UOD^{-1} hr^{-1}
k_La =Volumetric oxygen transfer coefficient, hr^{-1}
K_S =Monod's constant, mg/ml
n' =dilution factor
N $=C_X/C_{X_0}$
OD_a =empirical correlation factor
OD_b =optical density of the suspension before fermentation
OD_m =measured optical density after dilution
pH_s and pH_b=values of pH in interface and bulk respectively
t =fermentation time, hr
T =absolute temperature

Y_S =yield constant, UOD/mg

Greek symbols

α $=(C_{S_0}Y_S/Y_{X_0})+1$
α' =constant
β $=K_SY_S/C_{X_0}\mu_m$
ν $=1/\mu_m$
μ_m =maximum specific growth rate, hr^{-1}
ε =electronic charge
ψ =potential due to charged bacterial cells at the interface

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