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Effects of Water Activity on Biooxidation Kinetics

Utjecaj aktivnosti vode na kinetiku biooksidacije

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Summary

The effects of D-sorbitol concentration on water activity and the other parameters which determine the oxidation rate of D-sorbitol to L-sorbose when applying ***Gluconobacter suboxydans*** *S-22 as biocatalyst were studied. Oxygen solubility in sorbitol solutions, sorbitol solutions viscosity, water activity and diffusion permeability for different particles were determined. Water activity was defined on the basis of oxygen solubility. The effects of different parameters on kinetics of sorbose formation and microorganism growth were analyzed. The following was established: 1) The specific rate of sorbose formation is proportional to: a) specific rate of oxygen uptake, b) diffusion coefficient, c) reciprocal viscosity, d) difference between genuine and critical water activity; 2) Water activity affected microorganism growth more than sorbitol oxidation and critical water activity was found higher for growth than for bioconversion; 3) D-sorbitol can be converted into L-sorbose even when a part of sorbose crystallizes.*

Sažetak

Proučavan je utjecaj koncentracije D-sorbitola na aktivnost vode te druge činitelje koji određuju brzinu oksidacije D-sorbitola u L-sorbozu primjenom biokatalizatora ***Gluconobacter suboxydans*** *S-22. Određena je topljivost kisika u sorbitolnim otopinama te viskoznost, difuzijska propusnost i aktivnost vode tih otopina. Aktivnost vode definirana je na temelju topljivosti kisika. Analiziran je učinak različitih parametara na kinetiku tvorbe sorboze i rasta mikroorganizma. Ustanovljeno je: 1) da je specifična brzina tvorbe sorboze proporcionalna a) specifičnoj brzini potrošnje kisika, b) difuzijskom koeficijentu, c) recipročnoj viskoznosti i d) razlici pripadne i kritične aktivnosti vode; 2) da aktivnost vode jače utječe na rast mikroorganizma nego na pretvorbu sorbitola u sorbozu, a utvrđena kritična aktivnost vode veća je za rast nego za biokonverziju; 3) da se oksidacija D-sorbitola u L-sorbozu može zbivati i u uvjetima kada dio sorboze kristalizira.*

Introduction

The most frequently, biooxidation kinetics appears to be the function of four key factors – microorganism (biocatalyst) concentration, substrate concentration, temperature, and mass transfer rate. However, in the range of very high concentration of dissolved substances the water concentration, i.e. its activity can become the most important factor. As it is well known, the water plays dual role in biocatalytic reaction systems: a) it is required to form and maintain the native, catalytically active conformation of enzyme molecules, and b) most reactions in protein molecules resulting in enzyme inactivation, in particular thermoinactivation, also require water *(1)*. In the case of enzymatic oxidation of ethanol in the gaseous phase the alcohol oxidase activity was observed to increase with increasing water activity, while thermostability of the mentioned enzyme was observed to decrease with increasing water activity and vice versa *(1)*.

In living cells, and hence in microorganisms, effects of water activity depend on cell properties; for their growth bacteria commonly require high water activity (0.9–0.999) while some moulds and thermophile yeasts can grow in conditions of very low water activity (0.6) *(2)*. In nutrient media water activity depends on water concentration as well as on the concentration of other substances which by their different affinity bind the water, decreasing its availability and causing the vapour pressure of the solution decrease and osmotic pressure increase. This is the basis for water activity definition and determination methods, i.e. it is considered that

$$a_w = \frac{p_s}{p_w} \qquad /1/$$

and

$$\pi = \frac{-RT \ln a_w}{V_w} \qquad /2/$$

where: a_w = water activity, p_s = solution vapour pressure, p_w = vapour pressure of pure water, π = osmotic pressure of solution, V_w = molar volume.

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The process of microbial oxidation of D-sorbitol to L-sorbose can be considered as a convenient model to study the effects of different parameters on biooxidation kinetics, and therefore the effects of water activity can be evaluated as well. A special convenience of the mentioned process model is that it itself is of high importance since L-sorbose is an intermediate in L-ascorbic acid (vitamin C) production, and studies of L-sorbose production kinetics can lead to an improved vitamin C production process. Mori et al. *(3)* studied D-sorbitol to L-sorbose oxidation kinetics applying fed batch culture of *Gluconobacter suboxydans*. In their studies a special emphasis was placed on water activity in the fermentation broth. They established that L-sorbose of 628 g/L was attained after 14 hours of fed batch cultivation when water activity in the fermentation broth was found to be very low (0.68).

Our recent studies *(4)* showed that the oxidation process can be performed under cultivation conditions at which the formed sorbose partly precipitates, and that the total L-sorbose concentration in the fermentation broth can reach values above 800 g/L when more than 200 g/L refers to crystalline L-sorbose. The effect of D-sorbitol concentration on oxygen solubility was studied as well *(4)*. Experimental data induced the hypothesis that water activity could be defined on the basis of oxygen solubility. The aim of this work was to investigate more fully the effects of substrate on process kinetics in order to evaluate the significance of particular process parameters, as well as to define relationships between parameters, especially with respect to water activity defined on the basis of oxygen solubility.

Materials and Methods

Microorganism: Gluconobacter suboxydans S-22 (Culture Col. of PLIVA, Res. Institute, Zagreb).

Media:

1) Sterile water solutions of different D-sorbitol concentration;

2) Sterile media containing: CSL-filtrate – 20 g/L; D-sorbitol – various; water up to 1 L.

Cultivation method: Erlenmeyer flasks of 500 mL containing 50 mL of reaction media inoculated with *G. suboxydans* culture were shaken on a rotary shaker.

Analytical methods: 1) Diffusion rate assays: Petri dishes of 90 mm diameter with agarised D-sorbitol solutions of the composition: agar (Difco, Detroid, USA) – 3 g/L, bromo-phenol red (The British Drug Houses LTD., B.D.H. Laboratory Chemicals Group, England) – trace, D-sorbitol (PLIVA, Zagreb, Croatia) – various (i.e. containing 100, 200, 300, 400, 500, 600, 700 and 800 g/L, respectively) and water up to 1 L, were applied. Each was filled up with 20 mL of solution to form a layer of 3 mm thickness. A hole of 9 mm diameter in each agar layer center was bored and then filled with 1 M solutions of HCl, NaOH, lactic acid or D-gluconic acid containing the same concentration of D-sorbitol as that in the corresponding agarised layer. Changes of red (or yellow) colored zone diameter were followed and the data used to calculate diffusion coefficients applying the known method *(5)*.

2) Viscosity determination: rotation cylinder method and the apparatus »Rotavisco Haake RV3« (Berlin – – Karlsruhe, Germany) were applied. For given sorbitol solutions viscosity measurements at temperatures of 30 °C and 37 °C were performed.

3) Determination of oxygen uptake and sorbose formation rates:

a) The measuring device composed of the chamber designed as recommended by Krieg *(6)*, connected with a monitor (Biological oxygen monitor, model 5300, with Clark type polarographic micro electrode, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, USA) and a computer (Hewlett-Packard France, PC 486, Grenoble, France) was used to determine specific oxygen uptake rates. Upon saturation with oxygen of 35 mL of given sorbitol solution in the chamber, 1 mL of microorganism suspension (microorganism conc. 2 g/L) was injected into the chamber and then the change of dissolved oxygen followed. Taking into account the oxygen solubility and the rate of relative dissolved oxygen concentration decrease the specific oxygen uptake rates were calculated.

b) Sorbose formation rates were estimated on the basis of the measured changes of sorbose concentration in shake flask cultures. Autoanalyser »TRAACS 800« (Bran und Luebbe GmbH, Norderstedt, Germany) was applied for measurements. Mean specific sorbose formation rates were calculated applying the formula

$$q_P = \frac{2(\gamma_{Pi} - \gamma_{Pi-1})}{(t_i - t_{i-1})(\gamma_{Xi} + \gamma_{Xi-1})} \qquad /3/$$

c) Specific growth rate estimation: changes of microorganism concentration in shake flask cultures were followed by absorbance determinations at 660 nm (after sample dilution 1:3). Mean specific growth rates were calculated applying the formula

$$\bar{\mu} = \frac{2(\gamma_{Xi} - \gamma_{Xi-1})}{(t_i - t_{i-1})(\gamma_{Xi} + \gamma_{Xi-1})} \qquad /4/$$

In mathematical formula q_P = mean specific sorbose formation rate, $\bar{\mu}$ = mean specific growth rate, γ_P = sorbose mass concentration, γ_X = microorganism concentration and t = cultivation time; i = ordinal number referring to culture sample.

Additional data: recently published own data *(4)*.

Results

Table 1 gives some evidence of the properties of sorbitol solutions. In part the presented data have already been analyzed *(4)* and some relationships established, e.g. it was found that expressions

$$\gamma_{DO}/(\text{mg / L}) = 6.865 - 0.01208\,\gamma_S/(\text{g / L}) + 6.954 \cdot 10^{-6}\,(\gamma_S/(\text{g / L}))^2 \qquad /5/$$

and

$$\gamma_{DO}/(\text{mg / L}) = \frac{6.7822}{1.001867^{\gamma_S/(\text{g / L})}} \qquad /6/$$

describe well the oxygen solubility as a function of D-sorbitol concentration in solution. If the free water of solution is considered to be proportional to oxygen solu-

bility then its concentration could be determined by applying the following relation

$$\gamma_{w(}/(\mathrm{g}/\mathrm{L}) = \frac{996}{6.86}\gamma_{l(}/(\mathrm{mg}/\mathrm{L}) = \frac{984.7}{1.00867^{\gamma_{l(}/(\mathrm{g}/\mathrm{L})}} \qquad /7/$$

In concentrated media mass transfer could become a process limiting factor. Since mass transfer depends on diffusion, the effect of sorbitol concentration on diffusion rate of different particles was investigated applying the agar plate technique. The data are presented in Table 2, and as expected, they confirm the applicability of Fick's law. Numerical values of diffusion coefficients are increasing with increasing temperature and water activity of the solution, as well as with decreasing molecular mass of diffusible substances (e.g. for tested diffusible substances diffusion rates could be classified as follows: $H^+ > OH^- > lactate^- > D\text{–}gluconate^-$). In fact, medium viscosity is the main factor which determines diffusion rates of given particles through the medium, as clearly demonstrated in Fig. 1. Reciprocal values of solutions viscosities appear to be the linear function of free water concentration, whereas diffusion coefficients are proportional to reciprocal viscosities.

Oxygen uptake rate is a function of both the microbial biomass concentration and physiological state, and of oxygen availability. At low microbial biomass concentration one can consider the oxygen uptake rate to be low and practically constant during given experimental conditions in a measurement chamber and in the shake flask cultures. After plotting specific oxygen uptake rates observed in the chamber against the corresponding specific sorbose formation rates observed in shake flask cultures, the relationships presented in Fig. 2 were obtained. The data clearly show the specific sorbose formation rate to be proportional to the specific oxygen uptake rate. Proportionality factor could mean that only 74 % of consumed oxygen refers to the oxidation of D-sorbitol into L-sorbose. However, since compared data refer to different experimental systems such a conclusion should be verified by a new experiment where the data would refer to the same system. Confirmation of the mentioned finding would mean that values of volumetric oxygen transfer rate coefficient would be 35 % higher than those roughly estimated *(7)*, if the partial oxygen uptake directly from air bubbles could be neglected.

Table 1. Properties of D-sorbitol solutions (experiment temperature 28–29 °C)
Tablica 1. Svojstva otopina D-sorbitola (pri 28–29 °C)

Sorbitol concentration (g/L)	Density (g/L)	Oxygen solubility (mg/L)	Total water concentration (g/L)	Free water concentration (g/L)	Relation: free water/total water
0	996	6.86	996	996	1
100	1030	5.84	930	851	0.915
200	1061	4.51	861	657	0.763
300	1098	3.86	798	563	0.706
400	1132	3.10	732	452	0.617
500	1164	2.68	664	391	0.589
600	1193	2.13	593	310	0.523
700	1225	1.88	525	274	0.522
800	1257	1.54	457	224	0.490

Table 2. Diffusion permeability of D-sorbitol solutions and diffusivity of diffusible substances
Experimental system: Petri dishes with agar sorbitol layer, bored hole in the agar layer center filled with diffusible substance dissolved in sorbitol solution
Tablica 2. Difuzijska propusnost otopina D-sorbitola i difuzivnost difuzivnih tvari
Eksperimentalni sustav: Petrijeve zdjelice sa slojem agarizirane sorbitolne otopine, izbušena rupa u središtu agarnog sloja napunjena difuzionom tvari otopljenom u sorbitolnoj otopini

Temperature (°C)	Sorbitol concentration (g/L)	Diffusible substance	Diffusion rate coefficient (mm^2/min)	Diffusible substance	Diffusion rate coefficient (mm^2/min)
22	0	HCl	3.1068	NaOH	2.4760
	100		2.5889		1.3643
	200		2.0053		0.9432
	300		1.5469		0.6889
	400		1.0516		0.5167
	500		0.7181		0.3326
	600		0.4835		0.2316
	700		0.2278		0.1094
	800		0.1512		0.0765
	100	Lactic acid	1.0202	Gluconic acid	0.8367
37	100	HCl	2.8348	Lactic acid	1.3242
		NaOH	1.4688	Gluconic acid	1.0985

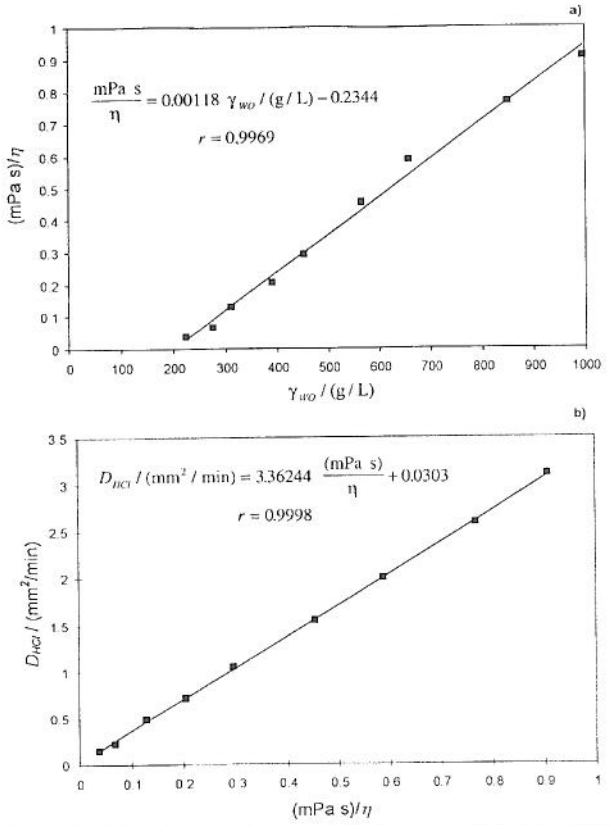

Fig. 1. a) Effect of free water concentration on solution viscosity, b) Diffusion-viscosity relationship
Slika 1. a) Utjecaj koncentracije slobodne vode na viskoznost otopine, b) Odnos viskoznosti i brzine difuzije

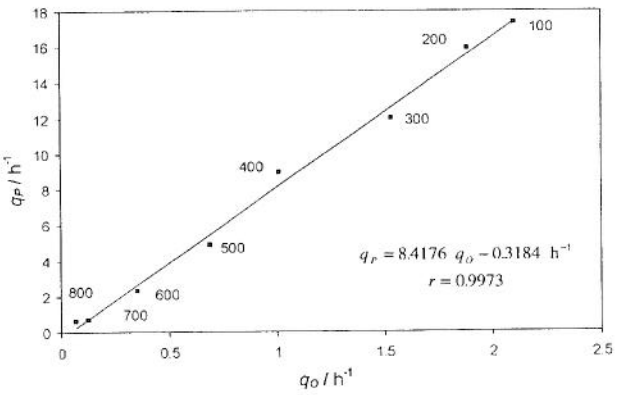

Fig. 2. Influence of D-sorbitol concentration (g/L; marked numerically) on specific oxygen uptake rate (q_O) and sorbose production rate (q_P)
Slika 2. Utjecaj koncentracije D-sorbitola (g/L; označeno brojevima) na specifičnu brzinu potrošnje kisika (q_O) i specifičnu brzinu tvorbe sorboze (q_P)

Relating the sorbose formation rate to sorbitol concentration, total and free water concentrations, and to medium viscosity and diffusion permeability, gave the relationships illustrated in Figs. 3a, b, c, d. The following is evident:

a) At sorbitol concentration range above 100 g/L both specific oxygen uptake rate and specific sorbose formation rate (Fig. 2 and Fig. 3) decreased with increasing sorbitol concentration;

b) Above a critical free water concentration the increase of specific sorbose formation rate appeared to be proportional to free water concentration increase;

c) Specific sorbose formation rate appeared to be directly proportional to the diffusion coefficient or reciprocally proportional to the medium viscosity.

Effects of sorbitol and water concentration on microorganism specific growth rate were also investigated and the results are presented in Fig. 4. As shown, the effects were stronger on microorganism growth (Fig. 4) than on sorbose formation (Fig. 3) and higher critical value of water activity (free water concentration) was established as necessary for growth.

Discussion

As it is well known *(6,7)*, in the common batch process of L-sorbose production the oxidation rate is primarily limited by microorganism concentration, then by oxygen transfer rate, and finally by sorbitol concentration. The process can be improved by inoculating media with more viable microbial cells and by biomass recycling *(8,9)*, by aeration – agitation intensification and/or by enriching the aeration stream with oxygen *(3,7)*, as well as by feeding the culture with D-sorbitol, i.e. by applying the fed batch culture methods *(3,4,6)*. Because of culture feeding with sorbitol the total concentration of sorbitol and sorbose increases, causing a simultaneous decrease of water activity, which can become even process limiting. Since in sorbose-saturated water solutions the water concentration, i.e. water activity, is higher than the critical value for sorbitol, one can expect that D-sorbitol can be converted into L-sorbose in conditions where sorbose formation becomes accompanied with simultaneous partial sorbose crystallization, as already experimentally established *(4)*.

In light of the presented data and the foregoing discussion the following conclusion can be drawn:

a) With increasing sorbitol concentration in reaction media, the oxygen solubility, water activity and diffusion permeability decrease, while media viscosity increases;

b) Diffusion of dissolved particles takes place according to Fick's law;

c) Numerical values of diffusion coefficients as well as of reciprocal viscosity are the linear functions of water activity, i.e. in the range where water activity is higher than critical, the values are proportional to the difference between genuine and critical water activity;

d) Specific rate of sorbose formation appears to be proportional to: a) specific oxygen uptake rate; b) diffusion coefficient; c) reciprocal viscosity, and d) difference between genuine and critical water activity;

e) Critical water activity appears to be higher for microorganism growth (Fig. 4) than for sorbose formation (Fig. 3);

f) Water equivalent to the oxygen solubility (free water concentration) turned out to be an appropriate parameter in expressing the water activity, i.e. it appears that the expression

$$a_w = \frac{\gamma_{WO}}{\rho^*_{WO}} \qquad /8/$$

could be applied.

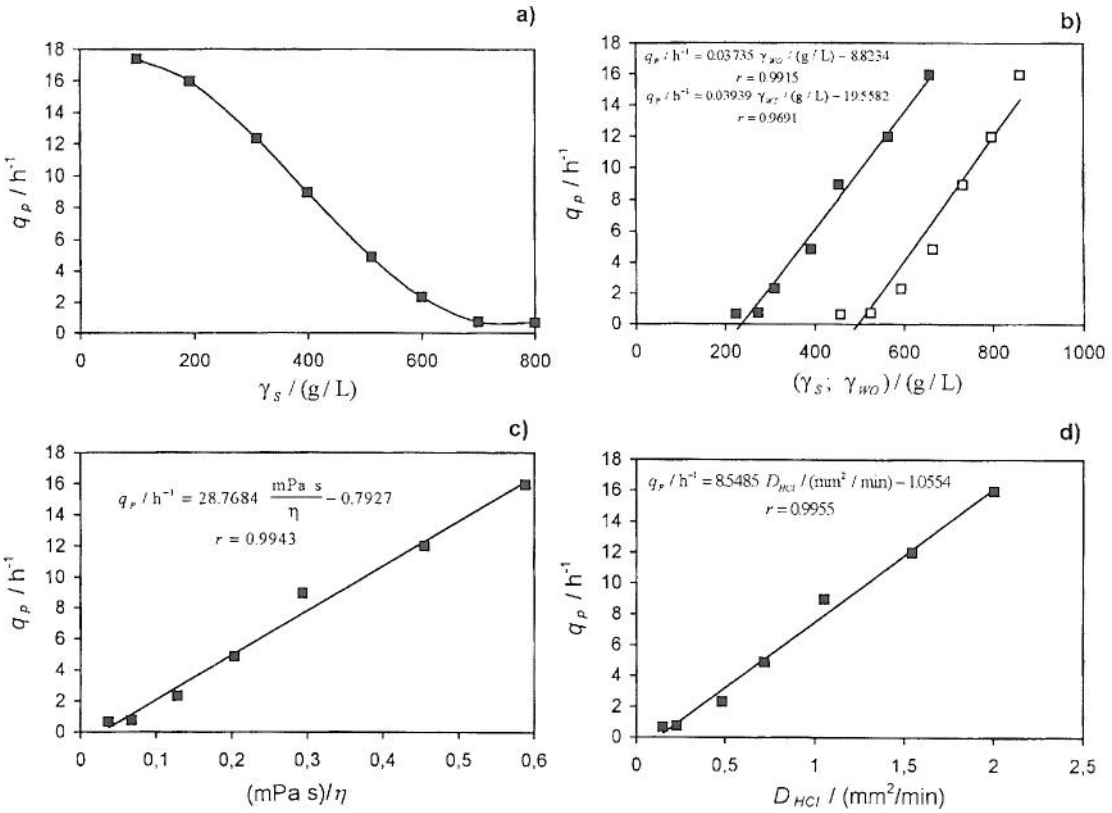

Fig. 3. The specific sorbose production rate as a function of a) sorbitol concentration, b) total and free water concentration, c) medium viscosity and d) medium diffusion permeability (diffusion coefficient)
Slika 3. Specifična brzina tvorbe sorboze kao funkcija a) koncentracije sorbitola, b) koncentracije ukupne i slobodne vode, c) viskoznosti medija i d) difuzijske propusnosti medija (koeficijenta difuzije)

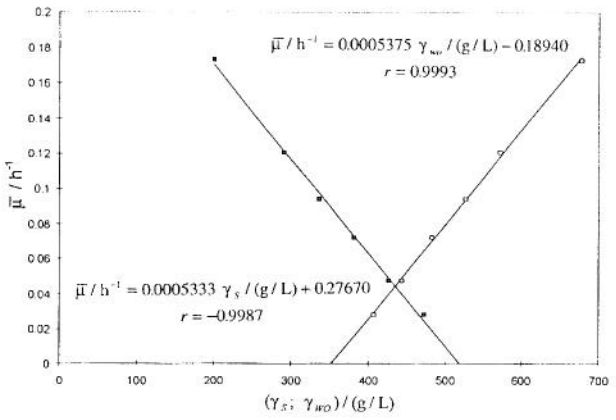

Fig. 4. Effect of D-sorbitol concentration (γ_S) and free water concentration γ_{WO}) on specific growth rate ($\bar{\mu}$)
Slika 4. Utjecaj koncentracije D-sorbitola (γ_S) i koncentracije slobodne vode γ_{WO}) na specifičnu brzinu rasta ($\bar{\mu}$)

g) D-sorbitol can be converted into L-sorbose even in reaction conditions where sorbose formation is accompanied by simultaneous partial sorbose crystallization.

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Symbols

a_w	water activity, dimensionless
D_{HCl}	diffusion coefficient referring to HCl, mm^{2}min^{-1}
i	ordinal number referring to culture sample, dimensionless
p_S	solution vapour pressure, Pa
p_w	vapour pressure of pure water, Pa
q_P	mean specific sorbose formation rate, h^{-1}
R	gas constant, J mol^{-1} K^{-1}
T	absolute temperature, K
t	cultivation time, h
V_w	molar volume, L mol^{-1}
γ_{DO}	disolved oxygen mass concentration, mg/L
γ_P	sorbose mass concentration, g/L
γ_S	sorbitol mass concentration, g/L
γ_{WO}	free water mass concentration, g/L
γ_X	microorganism mass concentration, g/L
η	viscosity, mPa s
$\bar{\mu}$	mean specific growth rate, h^{-1}
π	osmotic pressure of solution, Pa
ρ^*_{WO}	pure water density, g/L

References

1. E. Barzana, M. Karel, A. M. Klibanov, *Biotechnol. Bioeng.* 34 (1989) 1178–1185.
2. S. J. Pirt, *Principles of Microbe and Cell Cultivation*, Blackwell Sci. Publ., Oxford (1975) pp. 146–155.

3. H. Mori, T. Kobayashi, S. Shimizu, *J. Chem. Eng. Jpn. 14* (1981) 65–70.
4. M. Bošnjak, K. Mihaljević, *Med. Fac. Landbouw. Univ. Gent, 58/4b*, (Proc. 7th FAB, Gent 30 Sep. – 1 Oct., 1993) pp.1939–1948.
5. K. E. Cooper, The Theory of Antibiotic Inhibition Zones, in: Kavanagh, F., (Ed.) *Analytical Microbiology*, Academic, New York (1963) pp. 4–38.
6. P. Krieg, Doktors Natw. ETH Diss. Nr. 4675, Juris Druck + Verlag Zürich (1972).
7. M. Bošnjak, K. Gomerčić, P. Pintar, D. Mikačić, S. Matošić, *Chem. Biochem. Eng. Q. 2* (1988) 218–221.
8. D. N. Bull, M. D. Young, *Biotechnol. Bioeng. 23* (1981) 373–389.
9. M. Bošnjak, K. Mihaljević, N. Bošnjak, *6th Eur. Congr. Biotechnol.* (Firenze, June 13–17, 1993) Abs. books IV, TH093.