PRODUCTION OF CITRIC ACID BY FUNGI

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SUMMARY: A general review on citric acid production is written which cover only submerged fermentation processes. It contains some early literature which concentrates on fundamental aspects. There are many reports on the production of citric acid in submerged culture which show the wide variation in the conditions recommended for a successful fermentation. Since it has been shown that the nature and quantity of trace metals, carbon and nitrogen source and correct environmental conditions are very important for a successful citric acid fermentation, all these factors are discussed.

Key Words: A. niger, citric acid, fermentation.

INTRODUCTION

Citric acid is a 6-carbon containing tricarboxylic acid which was first isolated from lemon juice. It is a natural component of many citrus fruits, and was crystallized from lemon juice by Scheele in 1784. Approximately 70% of citric acid produced is used in the food and beverage industry for various purposes, 12% in pharmaceuticals and about 18% for other industrial uses (46). The estimated world production of citric acid was reported as 350.000 tons/year in 1986 (39). It however was recently reported that the world market requirement of citric acid is around 500.000 tons/year (6).

At the present day most citric acid is produced by fungal (*A. niger*) fermentation. Chemical synthesis of citric acid is possible but it is no cheaper than fungal fermentation. However, a small amount of citric acid, approximately less than 1% of total world production, is still produced from citrus fruits in Mexico and South America where citrus fruits are available economically. There are basically three different types of batch fermentation process used in industry. These are the Japanese koji process, the liquid surface culture and the submerged fermentation process (43), but nowadays nearly all citric acid is produced by submerged culture fermentations because profitability is relatively low and thus the economics of the operation are very constricted (6). Continuous fermentation has been studied on the laboratory scale by some workers (7,37,68,75) but no commercial production by this type of process is known. Since cell growth and citrate production occur at different times, and for the economic reasons, the substrate sugar must fully utilized, it is necessary to use multitask systems which would increase costs. In addition continuous culture usually gives lower citrate concentration i. e., recovery costs increase. Therefore continuous culture is probably not economically competitive at the present time.

MICROORGANISM

Although mainly *A. niger* has been used in the citric acid production process, other strains of fungi apart from *A. niger*, various kinds of yeast and some bacteria are known to accumulate citric acid in the medium (32). The reason for choosing *A. niger* over other potential citrate producing organism are: cheap raw materials (molasses) used as substrate; high consistent yields.

Inoculum

Steel *et al.* (67) recommended that between $120x10^3$ and $280x10^3$ pellets per liter (obtained from spore inoculated shake flasks) is a suitable inoculum level, although Kristiansen (36) indicated that the final concentration of citric acid and dry weight is not related to inoculum size, as long as it was kept below 10^6 spores/ml culture. Yigitoglu (75) therefore employed a less complex method for inoculum. Spores were harvested from PDA plates and incubated an orbital shaker at 30°C and 250 rpm for 48 hr. At the end of this period the small pellets which had formed in the flasks were used as inocula for fermenter experiments, using an inoculum level of 2%.

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Growth form

There is a general agreement in the literature that the pelleted form is desirable for acid production. An ideal pellet configuration, pellets of 1.2 to 2.5 mm diameter after five days, was described early (15). Gomez *et al.* (24) showed that the pelleted form is favorable due to pellet cultures have low culture viscosity causing improving bulk mixing and aeration conditions and lower oxygen consumption than in the cultures composed mainly of filamentous (dispersed) forms. Furthermore, problems of wall growth and pipe blockage are reduced and separation of biomass from culture liquid by filtration is considerably enhanced by the pelleted growth form.

Strain improvement

Strain improvement by mutation in order to achieve higher yields and higher trace metal tolerance is a continual aim of industrial producers. Its importance can be illustrated by the 500 fold increase in penicillin production from *Penicillium chrysogenum* due to mutation (33). But surprisingly little work has been published on increasing citric acid production by strain improvement. Probably production companies want to keep secret their successful methods. McKay *et al.* (51) increased the production of citric acid yields from glucose by *Yarrowia lipolytica* IFO 1658 two fold and by *Candida guillermondii* NRRL Y-448 from galactose, six fold via ultra-violet mutagenesis and subsequent selection. James *et al.* (29) produced a mutant via multiple X-ray and UV irradiation of spores, and mutant strains showing a six fold increase in citric acid yield compared to the parent strain. The success of a strain improvement programme is dependent on rapid and accurate screening. The method of Das and Roy (17) is a rapid method for correlating the acid producing ability of certain colonies. Martinkova *et al.* (49) concluded that the strain with improved citric acid production can be obtained by hybridization and they obtained 15% increase on term of citric acid production by isolated of the best heterozygous strains in comparison with the high-prototropic parent strain.

BIOCHEMISTRY OF CITRIC ACID OVER PRODUCTION

The biochemical pathways related to citric acid accumulation and the role of the tricarboxylic acid cycle (TCA) in fungi has been well established (46). Citric acid accumulation can be divided into three processes (39):

1. The breakdown of hexoses to pyruvate and acetyl-CoA by glycolysis,

2. Formation of oxaloacetate,

3. Condensation of acetyl-CoA and oxaloacetate to citric acid.

As citric acid synthesis involves the condensation of an acetyl unit with oxaloacetate, it is quite important to generate sufficient oxaloacetate in order for production to continue. Regeneration of oxaloacetate involves four mechanisms (32):

1. The direct carboxylation of pyruvate catalyzed by malic enzyme provides malate which is readily oxidized into oxaloacetate through malic dehydrogenase;

2. The carboxylation of pyruvate catalyzed by pyruvate carboxylase,

$$\text{Pyruvate} + CO_2 + H_2O + \text{ATP} \xrightleftharpoons{Mg^+} \text{Oxaloacetate} + \text{ADP} + \text{Pi}$$

3. The carboxylation of phospho-enol pyruvate (PEP) catalyzed by PEP carboxykinase,

$$\text{PEP} + \text{ADP} + CO_2 \rightleftharpoons \text{Oxaloacetate} + \text{ATP}$$

4. Via the glyoxylate cycle involving the key enzymes isocitrate lyase and malate synthase,

$$\begin{array}{l} \text{Isocitrate} \longrightarrow \text{Succinate} + \text{Glyoxylate} \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \text{Co-enzyme A} + \text{Malate} \rightleftharpoons \text{Glyoxylate} + \text{Acetyl-CoA} \\ \quad \downarrow \\ \text{Oxaloacetate} \end{array}$$

Pyruvate carboxylase is an important enzyme for citric acid production. It is poorly regulated, only weakly inhibited by 2-Oxoglutarate and not influenced by acetyl-CoA (30). Phosphofructokinase was the regulatory enzyme of citric acid production in *A. niger* (25). The enzyme was inhibited by high concentrations of citrate and ATP but activated by ADP, AMP, inorganic phosphate and ammonium ions. During citric acid production ammonium ions overcome the inhibition of PFK by citrate and ATP. Aconitase and isocitrate dehydrogenase are very important key enzymes in citric acid fermentation. The activity of these enzymes decrease to very low levels during the production stage which cause faulty operation of the cycle whilst the activity of citrate synthase increases (46).

FACTORS AFFECTING THE FERMENTATION PROCESS

Medium constituents

Trace elements: Trace element nutrition is one of the most important factors affecting the yields (grams citric acid per gram sugar) of citric acid fermentation. In

particular, the levels of manganese, iron, copper and zinc are quite critical. If the levels of these trace elements are correct other factors have less pronounced effects. Conversely, medium will not allow high production unless the trace element content is controlled carefully.

Manganese (Mn^{2+} ions) in the nutrient medium plays a key role in the accumulation of large amount of citrate by *A. niger*. When the Mn^{2+} concentration is maintained below 0.02 mM (which does not affect growth rate or biomass yield) large amounts of citric acid are produced (59). Clark *et al.* (12) observed 10% and 25% reduction in citric acid yield on adding 2 ppb and 100 ppb manganese to beet molasses respectively. Bowes and Matetey (5) also noted that by the addition of 10 ppm Mn^{2+} in the growth medium, citrate accumulation is effectively halved by *A. niger*. Orthafer *et al.* (54) found that manganese deficiency leads to significantly lower lipid content in *A. niger* whereas there were elevated lipid levels in manganese sufficient cultures. Jernejc *et al.* (30) stated that low mycelial lipid levels results in high yields of citric acid. Protein synthesis is also said to be inhibited by manganese deficiency (38).

Tomlinson *et al.* (69) found that up to 1 mg iron per liter medium is essential for high yields of citric acid by *A. niger*, but that amounts in excess of this interferes with citric acid accumulation. Partial deficiency of iron has also been reported to be necessary for citric acid production by several workers (9,62,66). The presence of excess iron favors the production of oxalic acid (42).

Copper ions play an important role in reducing the deleterious effect of iron on citric acid production (43, 69). It has also been reported that copper ions can successfully counteract addition of manganese to citric acid fermentation media and are inhibitors of cellular manganese uptake (39). Jernejc *et al.* (30) found that copper is an essential requirement for citric acid production and optimum concentration of Cu^{2+} is 40 ppm for high yield.

Low concentrations of zinc in the fermentation medium are generally favored in most citric acid production media (69). Chesters and Rolinson (9) reported that zinc deficiencies promote citric acid production. According to Wold and Suzuki (71), zinc plays a role in the regulation of growth and citrate accumulation. At high zinc levels (about 2 μM) the cultures are maintained in growth phase, but when the medium becomes zinc deficient (below 0.2 μM) growth is terminated and citric acid accumulation begins. Addition of zinc to citrate accumulating cultures results in their reversion to growth phase.

Since molasses (beet or cane) contains inhibitory amounts of metal ions like zinc, iron, copper, it is absolutely necessary either to remove these ions or to render them ineffective by pretreatment. The most commonly used methods of pretreatment are the addition of ferro or ferricyanide to precipitate iron, zinc, copper and manganese (22), decreasing the available manganese content to below 0.002 ppm (27) or passing the medium over ion exchange resing (42).

Sugars: Due to their rapid assimilation by fungus the usual carbon sources are glucose, fructose, or sucrose for high final yield of citric acid. For strain *A. niger* B60, maltose and sucrose, two disaccharides, were found to be better carbon sources for production of citric acid than the monosaccharides glucose and fructose (72). Some sugars such as galactose and arabinose have been reported to inhibit citric acid production (28, 45). In most cases, sucrose or its cheaper commercial source molasses is used. Several sources of crude carbohydrates have been used for citric acid production. e.g., date syrup, cotton waste, whey permeate, brewery waste, beat and cane molasses, unrefined sucrose, cane juice (39), grape pomace (26), filtered cheese whey with 2% methanol added (8) and banana extract (61) as a novel substrate for production of citric acid. Not only the type but also the concentration of the carbon source is important in the citric acid fermentation. Shu and Johnson (63) showed that a maximal citric acid production rate is obtained at 14 to 22% of sugar in the medium. The results of Anderson *et al.* (1) are in good agreement with those of Shu and Johnson. They found that 2, 6 and 10% sugar solutions give poor yields whilst getting high yield with 14, 18 and 20% sugar solutions. Xu *et al.* (72) also indicated that the optimal initial concentration of sucrose was around 10-14%. Begum *et al.* (3) found that combination of two sugars in the medium at 50% of each improved the yields of citric acid for the sucrose-glucose; glucose-sorbital; glucose-xylose and xylose-sorbital combinations with the mutant strains.

Nitrogen source: Usually ammonium sulfate or ammonium nitrate has been used as a nitrogen source. Physiologically, acid ammonium compounds are preferred since their consumption lowers the pH of the medium to below 2 which is an additional prerequisite of citric acid fermentation. Xu *et al.* (73) investigated the effect of different nitrogen sources on citric acid production by *A. niger* and produced 32 kg per m^3 citric

acid using urea. They also noted that the optimal concentration of ammonium sulfate was 5 kgm^{-3}. However Yigitoglu *et al.* (78) have found that the best initial ammonium sulfate level was 3 kgm^{-3} by a series of fermentation was carried out at varying initial concentration of ammonium sulfate between 0.5 and 4 kgm^{-3} in a 10 dm^3 STR. According to results of Choe and Yoo (11) the maximum cell mass and the maximum citric acid concentrations were obtained the initial ammonium nitrate concentration was 3 kgm^{-3} Choe and Yoo (11) have also found a relationship between the intracellular nitrogen source and citric acid biosynthesis. When the concentration of intracellular ammonium ion was between 2 and 3 mmol/g cell the production rate of citric acid was the highest. However when the concentration of intracellular ammonium ion decreased below 1 mmol/g cell, the citric acid production was stopped.

Phosphate: Shu and Johnson (64) reported that the effect of phosphate is not very pronounced but the balance between manganese, zinc and phosphate was critical. In any cases of trace metal contamination, phosphate limitation can have a beneficial effect on citric acid yield. Requirement of phosphate for fungal growth is 0.1 to 0.2% (32). However the presence of copper in the medium could reduce the optimum phosphate concentration (30). Martin and Steel (48) found that phosphate plays a key role in secondary metabolite production. When they added 0.005% phosphate to beet molasses, it was found that 5-ketogluconic and gluconic acid replace oxalic acid as secondary products. In addition fermentation time was significantly reduced.

Magnesium: Magnesium is essential for growth and citric acid production due to its role as a cofactor in a number of enzyme reactions in the cell. It has been reported that the optimum concentration of magnesium sulphate to produce maximum citric acid varies from 0.02 to 0.025% (32).

Environmental conditions

Aeration: Aeration has been shown to have a critical effect on the submerged citric acid production process. Khan and Ghose (34) suggested that aeration should be 0.6 vvm (liter air per liter medium per minute). The citric acid concentration was however raised from 30.3 to 48.7 kgm^{-3} by increasing air flow rate from 0.9 to 1.3 vvm (24). Clark and Lentz (14) found that citric acid production was related to oxygen pressure. The yield of citric acid increased by increasing the flow rate of air and the oxygen pressure up to 1.7 atmospheres using pure oxygen for pressures of 1 atm and greater, beyond which citric acid production would decrease. Batti (2) reported that interruption in aeration for one hour after 89 hours resulted in a 50% reduction in citric acid yield. The deleterious effect of interruption of aeration can be prevented by raising the pH to 3 until the production of citric acid commences again. Dawson *et al.* (18) observed that 20 min interruption in aeration, at fermentation time 1 and 3 days, had slightly inhibitory effect on acid production until forty day. This effect however was quickly overcome since DOT (dissolved oxygen tension) never fell below 20% of saturation, which is well above the critical value of 12-13% and had no effect on the final citrate production and mycelial growth. When the air supply to the fermentation was interrupted for 120 minutes on days 1 and 3, DOT value decreased to zero, mycelial growth and citric acid production, but not sugar consumption, ceased. However the effect was not permanent and both the growth and acid production rates eventually recovered.

Agitation: Clark and Lentz (13) found that agitation in stirred tank fermenters was critical. Increasing agitator speed would break up pellets, leading to dispersion of more than 95% of pellets, resulting in higher yields of citric acid. Maximum yield was obtained at agitator speeds between 400 and 700 rpm. Khan and Ghose (34) also reported that 500 rpm was the optimal agitation speed for citric acid production. However Roukas (60) claimed that the highest yield of citric acid 28 kgm^{-3} was obtained in culture agitated at lower impeller speed (300 rpm). The activity of citrate synthase decreased with the increase of speed of agitation while activity of aconitase and isocitrate dehydrogenase increased with the increase in agitation speed. Gomez *et al.* (24) found that the length of the fermentation for any given agitator speed was important. They produced 48 kgm^{-3} citric acid when carrying out fermentation at l000 rpm, but the concentration of citric acid was only 30.3 kgm^{-3}, applying agitation speed 450 rpm for the first 48 h, and 1000 rpm for the best of the fermentation period.

pH: There is no agreement in the literature about optimal initial pH. Moyer (53) found that ph did not have any effect within the range 1.95 to 3.10. Khan and Ghose (34) however reported that citric acid yield increased with increasing pH. The optimal initial pH was

found to be 6.5. On the other hand, optimal initial pH was been reported to be 2.8 and 2.5 by Kamal and Gupta (31), Jernejc *et al.* (30) respectively. However in the last two studies the work was carried out in shake flask cultures with the usual associated drawbacks, thus it is often difficult to make valid comparisons. Yigitoglu *et al.* (78) have indicated that a pH of 2.5 is a clear optimum for final product concentration in a 10 dm^3 STR. Charley (7) reported that the optimal final pH for batch fermentation was 1.7. This was supported by the findings of Kubicek and Rohr (39) which recommended that pH should be kept low (below 2.0). According to that report at higher pH's, *A. niger* accumulates gluconic acid, especially when the pH is around 4.0 (39). Marison (46) also reported that pH should be maintained around 2.0. Yigitoglu *et al.* (78) however reported that culture pH should be controlled between 1.8 and 2.5.

Incubation temperature: According to Steel *et al.* (67) the incubation temperature should be in the range to 28 to 32°C, while Gerhardt *et al.* (22) found that 30°C was the optimum for citric acid production. Khan and Ghose (34) also reported that 30°C was the optimum for citric acid production.

Duration of fermentation: It has been reported that the citric acid fermentation is completed in 8 days. Extension of the fermentation period did not increase the yields of citric acid (55). Kamal and Gunta (31) also obtained maximum yield with incubation period of about 6-9 days.

Other factors

Alcohols: Moyer (53) found that addition of lower alcohols, methanol, ethanol, n-propanol, to crude carbohydrate raw materials could increase the yield of citric acid. Optimal concentration of methanol, which was said to be more effective than ethanol, varied from 1 to 4% by volume. However addition of methanol to highly purified, high yielding substrates is deleterious to acid yields. Hang and Woodams (26) observed that the presence of methanol at a concentration of 3% in grape pomace led to an increase in the citrate yield by a factor of around 3. The exact mechanism of the alcohol effect however is unexplained, though it is postulated (46) that addition of methanol increases the tolerance of fungi to Fe^{2+}, Zn^{2+} and Mn^{2+}.

Lipids: Milis *et al.* (52) found that addition of natural oils with a high content of unsaturated fatty acids and oleic acid at 2% (v/v) to fermentation media led to increases in the yield by 20% without affecting dry weight of mycelium. They suggested that unsaturated fatty acids act as an alternative hydrogen acceptor to oxygen during the fermentation, since only high levels of unsaturated fatty acids were effective in improving the yield of citric acid. Gold and Kieber (23) found that a concentration of fatty acid of 0.05 to 0.3% had to be maintained during the fermentation.

Vitamins: Lal and Srivastava (40) reported the effect of different concentration (range $1.0x10^{-5}$ to $5.0x10^{-5}$ M) of some vitamins on growth and citric acid production in sugar medium by *A. niger* AL 29. They observed that ascorbic acid and p-amino benzoic acid, at all concentrations, inhibited both growth and citric acid. Conversely, the presence of thiamine ($3x10^{-5}$ M) and riboflavin ($4x10^{-5}$ M) stimulated the citric acid formation to the extent of 59% and 50% respectively. Biotin ($3x10^{-5}$ M) produced the greatest enhancement stimulating growth and increasing the production of citric acid by 66.4%.

Amino acids: Lal and Srivastava (41) observed the effect of different concentration (range $1.0x10^{-3}$ to $5.0x10^{-3}$ M) of some amino acids on growth and citric acid production in sugar medium by *A. niger* AL 29. According to them the presence of glutamic acid ($4x10^{-3}$ M) and aspartic acid ($3x10^{-3}$ M) stimulated citric acid production by 79 and 76.7% respectively. Presence of lysine ($5x10^{-3}$ M) and serine ($4x10^{-3}$ M) also could influence the formation of citric acid by 62.3 and 50.4%. The effect of cysteine (in all concentrations) was found to be detrimental.

Toxic chemicals: Qureshi and Qadeer have studied (58) the effect op addition of 'toxic' chemicals (range 0 to 60 ppm) such as phenol, resorcinol, hydroquinone, o-cresol, x-naphthol and b-naphthol on spore germination, mycelial dry weight mand citric acid production in a based glucose medium using *A. niger* EU-1. There was slight increase in citric acid formation in the presence of phenol (20 ppm) and b-naphthol (20 ppm). But hydroquinone (with 30 ppm) and o-cresol (with 15 ppm) exhibited maximum citric acid stimulation i.e. 85 and 80 kgm^{-3} respectively. Acid formation in the presence of resorcinol (with 50 ppm) was 78 kgm^{-3}. They concluded that the increase in citric acid production may be due to either the direct effect of these phenols on the growth process i.e., metabolism of *A. niger*, or to the inhibition of enzymes involved in further metabolism of citric acid.

Ammonium sulphate and citrate supplementation: The effect of a single pulse of ammonium sulphate and citrate upon the progress and final outcome of batch citric acid fermentation was studied by Yigitoglu and McNeil (76). It was found that the optimum addition time of N was in the range of 40 to 75 h. It was found that final citric acid concentration achieved was increased when the amount of supplemental N source added was between 0.25 and 0.5 kgm^{-3}. 60 kgm^{-3} citric acid addition at time 90 h, led to a 40.3% increase in the final citric acid concentration (80 kgm^{-3}) compared to the standard run in which 57 kgm^{-3} citric acid was produced (79).

KINETICS OF GROWTH AND PRODUCT FORMATION

It has been suggested (57) that fungi grow in the filamentous form at an exponential rate with a constant specific growth rate (μ) until some substrate becomes growth limiting, according to the Monod equation:

$InX=InX_0+ \mu t \quad \mu\mu=\mu max\ (S) / Ks + S$

where μmax : The specific growth rate of the organism (in h^{-1}); Ks: Saturation constant (kgm^{-3}); S: The concentration of the limiting substrate (in kgm^{-3}) Emerson (19), however, proposed that the filamentous mode of growth could best be described by the assumption of cube root kinetics:

$X^{1/3} = X_0^{1/3} + Kt$

where X_0 is cell present at t = 0; K: Constant.

Yano *et al.* (74) suggested that the cube root model was applicable to pellet growth due to the diffusion of oxygen into the pellet being the growth limiting step. Marshall and Alexander (47) obtained a linear relationship between the cube root of oxygen consumption and time for a number of fungi, including *A. niger*. Pirt (56) suggested that cube root kinetics was valid for growth of fungi in pellets, whilst exponential growth could be associated with the fungus growing in the filaments. Trinci (70) found that *A. nidulans* grew exponentially until the pellets reached a certain thickness, after which the cube root model was followed. Borrow *et al.* (4) obtained a similar result for the growth of Giberella fujikuroi under nitrogen limitation, growth changed from exponential to linear and then cube root. Kono and Asai (35) found that different models could be modeled to describe the growth kinetics an organism of different stages in the batch fermentation. The general equation for growth is;

$dx/dt=K\ \Phi\ X$

where K is a constant, X is cell concentration (kgm^{-3}), Φ represents an apparent coefficient for growth. During the decline phase, endogenous respiration may become important so it should be taken port into the proposed model. Chiv *et al.* (10) proposed that the rate of decay could be modeled by a first order reaction, so cell growth became;

$dx/dt = \mu X - KaX$

where m is specific growth rate and Ka is a constant. A similar model was proposed by Sinclair and Topiwala (65).

Product formation in fermentations was classified by Maxon (50) and Gaden (21) indepently in 1955. Maxon related the product formation to cell growth, whereas Gaden related it to substrate utilization. Luedeking and Piret (44) was used the following expression to describe the product formation model;

$dP/dt= \propto dX / dt + \beta X$

where P= product concentration (kgm^{-3}), X= bimass concentration (kgm^{-3}), $\propto$ and β are constant. In some fermentations, usually when carbon becomes limiting, the organism will break down its product and use it as substrate. So Constantinides *et al.* (16) modified the Leudeking-Piret model to account for this;

$dP/dt= \alpha dX / dt + \beta X - \gamma P$ where γ is a constant.

REFERENCES

1. Anderson JG, Blain JA, Divers M, Todd JR : Biotech Lett, 2, 3, 99, 1980.

2. Batti MA : US Patent, 3, 290, 227, 1966.

3. Begum AA, Choudhury N, Islam MA : J Ferm Bioeng, 70, 4, 286, 1990.

4. Borrow A, Brown S, Jefferys EG, Kessell RHJ, Lloyd EC, Lloyd PB, Rothwell A, Rothwell B, Swait JC : Can J Microbiol, 10:407, 1964.

5. Bowes I, Matetey M : FEMS Microbiol Lett, 6:219, 1979.

6. Bu'lock JD : Biotech Insight, 84, 5, 1990.

7. Charley RC : PhD thesis, University of Strathclyde, 1981.

8. Chen A, Liao PH, Lo KV : Can Agrcul Eng, 32, 2, 329, 1990.

9. Chesters CGC, Rolinson GN : Gen Microbiol, 5:553, 1951.

10. Chiv SY, Fan LT, Kao IC, Ericson LE : Biotech Bioeng, 14:129, 1972.

11. Choe J, Yoo YS : J Fermen Bioeng, 72, 2, 106, 1991.

12. Clark DS, Ito K, Horitsu H : Biotech Bioeng, 8:465, 1966.

13. Clark DS, Lentz CP : Biotech Bioeng, 5:193, 1963.

14. Clark DS, Lentz CP : Can J Microbiol, 7:447, 1961.

15. Clark DS : Can J Microbiol, 8:133, 1962.

16. Constantinides A, Spencer JL, Gaden EL Jr : Biotech Bioeng, 12:803, 1970.

17. Das A, Roy P : Advances in Biotech, 1:51, 1981.

18. Dawson M, Maddox IS, Brooks JD : Enzyme Microbiol Tech, 8:37, 1986.

19. Emerson SJ : Bact, 60:221, 1950.

20. Feir HA, Suzuki I : Can J Biochem, 47:697, 1969.

21. Gaden EL Jr : Chem Ind, p 154, 1955.

22. Gerhardt P, Dorrell WW, Baldwin IL : J Bact, 52:555, 1946.

23. Gold W, Kieber R : US Patent, 3, 373, 094, 1966.

24. Gomez R, Scnabel I, Garrido J : Enzyme Microbiol Tech, 10:188, 1988.

25. Habison A, Kubicek CP, Rohr M : FEMS Microbiol Lett, 5:39, 1979.

26. Hang YD, Woodams EE : Biotech Letters, 7, 4, 253, 1985.

27. Horitsu H, Clark DS : Can J Micromiol, 12:901, 1966.

28. Hossain M, Brooks JD, Maddox IS : App Microbiol, 22, 2, 98, 1985.

29. James LW, Rubbo SD, Gardner JF : J Gen Microbiol, 14:223, 1956.

30. Jernejc K, Cimerman A, Perdih A : Eur J Appl Microbiol Biotech, 14:29, 1982.

31. Kamal KSS, Gupta BK : Life Sci Adv, 1:89, 1982.

32. Kapoor KK, Chaudhary K, Tauro P : In "Prescott and Dunn's Industrial Microbiology" 4th edition, p 709, 1982.

33. Kelly W : In "Biotechnology for Engineers Biological Systems in Processes" p 219, 1988.

34. Khan AH, Ghose TK : J Ferm Tech, 51:734, 1973.

35. Kono T, Asai T : Biotech Bioeng, 11:293, 1969.

36. Kristiansen B : PhD thesis, UMIST, 1976.

37. Kristeansen B, Sinclair CG : Biotech Bioeng, 21:297, 1979.

38. Kubicek CP, Hampel W, Rohr M : Arch Microbiol, 123:73, 1979.

39. Kubicek P, Rohr M : CRC Critical Reviews in Biotech, 3, 4, 331, 1986.

40. Lal DN, Srivastava AS : Zbl Microbiol,137, 31, 1982.

41. Lal DN, Srivastava AS : Zbl Microbiol,137, 381, 1982.

42. Lockwood LB : In "The Filamentous Fungi", 1:140, 1974.

43. Lockwood LB : In "Microbial Technology, Microbial Processes", 1:335, 1979.

44. Luedeking R, Piret EL : J Biochem Microbiol Tech Eng, 1, 4, 393, 19.

45. Maddox IS, Spencer K, Greenwood JM, Dawson MW, Brooks LD : Biotech Lett, 7:815, 1985.

46. Marison IW : "Biotechnology for Engineers Biological Systems in Processes", p 323, 1988.

47. Marshall KC, Alexander M : J Bac, 80, 412, 1960.

48. Martin SM, Steel R : Can J Microbiol, 1:470, 1955.

49. Martinkova L, Musilkova M, Ujcova E, Machek E, Seichert L : Folia Microbiol, 35, 143, 1990.

50. Maxon WD : App Microbiol, 3:110, 1955.

51. McKay IA, Maddox IS, Brooks JB : In "International Biotech Conference on Fermentation Technologies: Industrial Applications", p 285, 1990.

52. Milis NF, Trompy BH, Palmer BM : J Gen Microbiol, 30, 365, 1963.

53. Moyer AJ : Appl Microbiol, 1:1,1953.

54. Orthafer R, Kubicek CP, Rohr M : FEMS Microbiol Lett, 5:403, 1979.

55. Palo ND, Cunanan LF, Danguilan ML : Philipp J Scn, 113, 1-2, 11, 1984.

56. Pirt SJ : Proc Royal Soc B, 66, 369, 1966.

57. Pirt SJ, Callow DS : Nature, 184, 307, 1959.

58. Qureshi MTH, Qadeer MA : J Pure and Appl Sciences, 6, 1, 17, 1987.

59. Rohr M, Kubicek CP, Kominek J : In "Biotechnology", 3:419, 1983.

60. Roukas T : J Industrial Microbiol, 7, 3, 221, 1991.

61. Sassi G, Ruggeri B, Specchia V, Gianetto A : Bioresource Tech, 37, 259, 1991.

62. Schweiger LB : US Patent, 2, 970, 084, 1961.

63. Shu P, Johnson MJ : Ind Eng Chem, 40, 1202, 1948.

64. Shu P, Johnson MJ : J Bac, 56, 577, 1948.

65. Sinclair CG, Topiwala HH : Biotech Bioeng, 12, 1069, 1970.

66. Snell RL, Schweiger LB : S Patent, 2, 491, 667, 1949.

67. Steel R, Lentz CP, Martin SM : Can J Microbiol, 1, 299, 1955.

68. Stevenson PM : PhD thesis, University of Strathclyde, 1986.

69. Tomlinson N, Campbell JJR, Trussell PC : J Bact, 59, 217, 1950.

70. Trinci APJ : Arch Microbiol, 73, 353, 1970.

71. Wold WSM, Suzuki I : Can J Microbiol, 22, 1093, 1976.

72. Xu DB, Kubicek CP, Rohr M : Appl Microbiol Biotech, 30, 444, 1989.

73. Xu DB, Madrid CP, Rohr M, Kubicek CP : Appl Microbiol Biotech, 30, 553, 1989.

74. Yano T, Kodamo T, Yamada K : Agr Biol Chem, 25, 580, 1961.

75. Yigitoglu M : PhD thesis, University of Strathclyde, 1992.

76. Yigitoglu M, McNeil B : Biotech Lett, 14, 9, 831, 1992.

77. Yigitoglu M, McNeil B, Kristiansen B : J Chem Tech Biotech, "Extended Summaries SCI Biotechnology Group 2nd Annual Students' Meeting on Fermentation Biotechnology, Effect of initial ammonium concentration on citric acid production in a stirred tank reactor", 4:297-305, 1992.

78. Yigitoglu M, McNeil B, Kristiansen B : J Chem Tech Biotech, "Extended Summaries SCI Biotechnology Group 2nd Annual Students' Meeting on Fermentation Biotechnology, Effect of pH on citric acid production in a stirred tank reactor" , 4:297-305, 1992.

79. Yigitoglu M, McNeil B, Kristiansen B : First UK Biotech Congress, G 10, 1991.

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