EFFECTS OF TRACE METALS AND MEDIUM COMPOSITION ON THE GROWTH OF *Aspergillus niger* ATCC 11414, IN A SUBMERGED CULTURE

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ABSTRACT

In an attempt to optimize citric acid fermentation, a study has been conducted to determine optimum nutritional conditions for the growth of ***Aspergillus niger*** *ATCC 11414. The objective of the study was to obtain information on the growth of this strain in a submerged culture for the study of citric acid production. The following article summarizes the results of study on the effects of trace metals and composition of chemically defined medium on mycelial growth of* ***A.niger*** *ATCC 11414. Chemically defined media containing glucose as the carbon source and energy were used throughout the work. Growth experiments were carried out by a submerged culture process, in a 300-ml Erlenmeyer flask which contained 50ml liquid medium. The process was conducted at 30°C for 4 days in an orbital shaker incubator operated at 200 rpm. The cultivation process was followed by monitoring the changes in the culture medium of the concentrations of biomass, total reducing sugars, citric acid, and pH of the medium. It was concluded that copper (II), iron (II), zinc (II), and manganese (II) ions had a remarkable effect on the growth of* ***A. niger*** *ATCC 11414. With 5% glucose, the study showed that 5 - 15 ppm copper, 0.5 - 25 ppm iron and 0.5 - 25 ppb manganese ions were optimal for the growth of the strain. The growth of the strain increased with the increase of* Zn^{2+} *added (0.5 - 25 ppm). The most optimal medium for the growth of* ***A.niger*** *ATCC 11414 was found to be able to produce more than 16 g of dry weight of biomass for 50 g glucose.*

INTISARI

Di dalam upaya mengoptimalisasi fermentasi asam sitrat, telah dilakukan penelitian untuk menentukan kondisi hara yang optimum untuk pertumbuhan ***Aspergillus*** *niger ATCC 11414. Penelitian dimaksudkan untuk memperoleh informasi tentang pertumbuhan A.niger yang dapat digunakan dalam produksi asam sitrat secara fermentasi biak-rendam. Medium racik-kimia yang mengandung glukosa sebagai sumber karbon dan energi telah digunakan dalam penelitian ini. Percobaan pertumbuhan dilakukan secara biak-rendam di dalam labu Erlenmeyer 300-ml yang mengandung 50 ml medium cair. Kultivasi dilakukan pada 30°C selama 4 hari, di dalam inkubator goyang-orbital dengan tingkat goyangan 200 rpm. Proses kultivasi diikuti dengan memantau perubahan medium untuk konsentrasi biomassa, gula-pereduksi total, asam sitrat serta tingkat pH medium. Dari hasil penelitian dapat disimpulkan bahwa ion tembaga (II), besi (II), seng (II) dan mangan (II) mempunyai pengaruh yang besar pada pertumbuhan* ***A.niger*** *ATCC 11414. Pada 5% glukosa, didapatkan bahwa 5 - 15 ppm tembaga, 5 - 25 ppm besi dan 5 - 25 ppb mangan adalah optimum untuk pertumbuhan* ***A.niger*** *ATCC 11414. Pertumbuhan strain tersebut meningkat dengan meningkatnya seng yang ditambahkan (0.5 - 25 ppm). Pertumbuhan* ***A.niger*** *ATCC 11414 yang optimal menghasilkan lebih dari 16 g biomassa pada 5% glukosa.*

INTRODUCTION

In citric acid fermentation, maximum yields require a proper balance between growth and citric acid formation. Cochrane [1] suggested that at some point adequate cell material should exist to convert the substrate with maximum efficiency into citric acid. Studies of the effect of biomass density on citric acid production rate in shake flask culture [2] also indicated that to some extent the rate of citric acid formation was proportional to the biomass level.

It follows that the knowledge of the growth of the producing strain is an indispensible prerequisite in any attempt to maximize citric acid production, especially by a two-stage fermentation process. In this process, the mould is firstly grown in a *growth medium* with a proper composition to produce the required biomass of the producing strain. The preformed mycelium is then recultivated in a proper *replacement medium* to produce citric acid.

In an attempt to optimize citric acid fermentation, a study has been conducted to determine optimal nutritional conditions for the growth of Aspergillus niger ATCC 11414.

Reports of study on the growth of A.niger were scarce, and the information concerning the effect of medium composition on the growth of A.niger was originally based on the studies reported by Steinberg [3- 9]. However the data of these growth studies were given in terms of biomass production at a fixed incubation time (4 days at 35°C) so that it would not be possible to evaluate the changes in the culture during the cultivation. Later, studies on kinetics of biomass production of A.niger in a one-stage citric acid fermentation process were reported by Berry et al. [10] and Roehr et al. [11].

The literature suggested that A.niger was able to grow in a simple artificial media containing sources of carbon, nitrogen, minerals and trace metals. However, nutritional requirements of the mould were highly specific, so that individual strain differed from each other both quantitatively and qualitatively in their nutritional conditions either for optimal cellular growth or citric acid production [1].

The following article summarizes the results of a study on the effects of trace metals and composition of chemically defined media on mycelial growth of *A.niger* ATCC 11414.

The objective of the study was to obtain information on the growth of the strain *A.niger* ATCC 11414 in a submerged culture for the study of citric acid production.

MATERIALS AND METHODS

Chemicals

The water used was demineralized one (resistance of 10 -18 Megaohm/cm) obtained from a Millipore demineralizer. All chemicals were of pro-analytical grade, and the trace metals employed were added in the form of their sulphate salts.

Organism

The organism used was *A.niger* ATCC 11414 which was obtained from The American Culture Collection. The mould was cultivated and maintained on Bacto-Potato Dextrose Agar (PDA) slant. After incubation at 30°C for 7 days, conidiospores of *A.niger* ATCC 11414 were harvested and suspended in sterile 0.005% Tween-80. This spore inoculum was used at the rate of 10^3 millions spores per-litre medium. The spore concentration of the inoculum was determined using a haemacytometer. The agar-slant culture was stored for 1 - 2 months (4°C), before it was recultivated on fresh PDA agar-slants.

Culture Media

The glucose media of the following compositions were used in the study.

Glucose medium A: Glucose (50 g/l), NH_4NO_3 (1.5 g/l), KH_2PO_4 (1.20 g/l), $MgSO_4.7H_2O$ (0.50 g/l), Fe^{2+} (25 ppm), Zn^{2+} (25 ppm), and Mn^{2+} (25 ppb).

Glucose medium B: Glucose (50 g/l), NH_4NO_3 (1.5 g/l), KH_2PO_4 (1.20 g/l), $MgSO_4.7H_2O$ (0.50 g/l), and Cu^{2+} (5 ppm). Unless otherwise stated, Fe^{2+}, Zn^{2+} and Mn^{2+} ions were respectively added at the level of 25 ppm, 25 ppm and 25 ppb.

Glucose medium G: Glucose medium G of different compositions (Table 1), were tested as the growth medium for *A.niger* ATCC 11414.

Table 1 Compositions of glucose medium G tested as growth medium for the submerged cultivation of ***Aspergillus niger*** ATCC 11414.

		Types of glucose medium G				
		G1	G2	G3	G4	G5
Glucose,	g/L	50	50	50	50	50
NH_4NO_3,	g/L	2.06	2.06	2.06	2.06	2.06
KH_2PO_4,	g/L	1.2	1.20	1.2	0.55	1.20
$MgSO_4.7H_2O$,	g/L	0.50	0.50	0.50	0.22	0.50
Trace metals ions:						
Cu^{2+},	ppm	5.0	5.0	0.05	0.05	0.05
Fe^{2+},	ppm	25.0	25.0	0.30	0.30	0.03
Zn^{2+},	ppm	25.0	25.0	0.20	0.20	0.02
Mn^{2+},	ppb	25.0	25.0	25.0	25.0	25.0
Mo^{6+},	ppb	-	20.0	20.0	20.0	20.0

Sterilization

Sterilization of the culture medium was done at 121°C for 15 minutes in an autoclave.

Culture Methods and Conditions

The chemically defined media as formulated above were used throughout the work. Growth experiments were carried out by a submerged culture process, in a 300-ml Erlenmeyer flask which contained 50ml of the liquid medium. The process was conducted at 30°C for 4 days, in an orbital shaker incubator operated at 200 rpm. Submerged culture cultivation runs were in duplicates, unless otherwise stated.

Assays

The cultivation process was followed by monitoring the changes in the culture medium of the concentrations of biomass, total reducing sugars, citric acid and the pH of the medium.

Biomass concentration was determined by dry-weight method of Roehr *et al.* [11], by drying the sample at 105°C to a constant weight (overnight). Glucose was determined as reducing-sugar by copper reduction method of Saeffer-Somogyi [12]. Citric acid was determined by the HPLC method using a μ-Bondapak column after the filtrate has been filtered with a Se-pak C_{18} cartridge. The pH of the medium was measured directly with a pH-meter.

RESULTS AND DISCUSION

Effects of trace metals on mycelial growth of *A.niger* ATCC 11414

Results of the study shown in Figure 1 clearly indicates that both glucose consumption and biomass production during the submerged cultivation of *A.niger* ATCC 11414 were poor in glucose medium A containing no added trace metals. No citric acid analysis was attempted in this case.

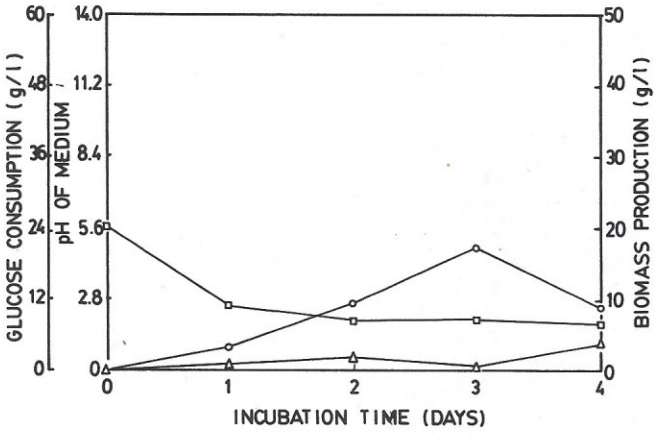

Figure 1. Growth and chemical changes in the course of cultivation of *A.niger* ATCC 11414 in glucose medium A with no added trace metals (Cu^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+} ions).
Δ Biomass production
○ Glucose consumption
□ pH of medium

Foster [13] pointed out that in an artificial media, the amount of trace metal ions required to obtain maximum growth of fungi were minute compared with other mineral constituents. Some of these metals, particularly copper, iron, zinc, manganese and molybdenum, have been identified to be essential and indispensible for the growth of *A.niger* [9,13].

In this study, the effect of these essential trace metals on the growth of *A.niger* ATCC 11414 were investigated and reported. This study revealed that copper, iron, zinc and mangenese all influenced the growth of this strain remarkably.

The results of a study on the influence of copper on the growth of *A.niger* ATCC 11414 are presented in Figure 2.

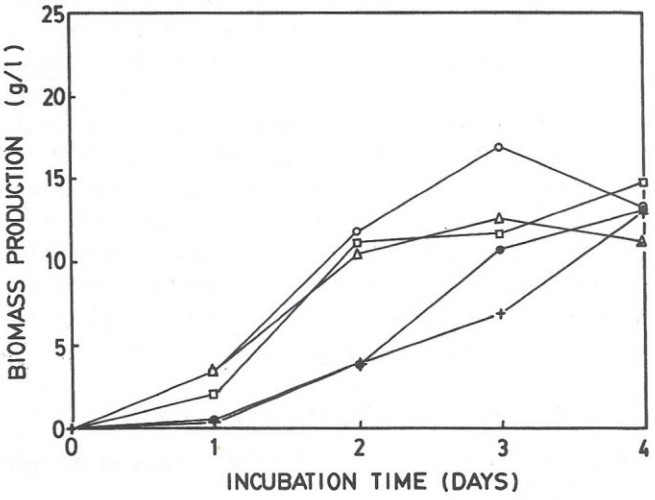

Figure 2. Mycelial growth of *A.niger* ATCC 11414 at various concentrations of Cu^{2+} ion, in medium glucose A.
○ Cu (5ppm), Δ Cu (10ppm), □ Cu (15ppm),
● Cu (20ppm), + Cu (25ppm)

The growth of the mould was then evaluated mainly on the values of the initial linear-growth rate, total biomass production and biomass yield (Figure 3).

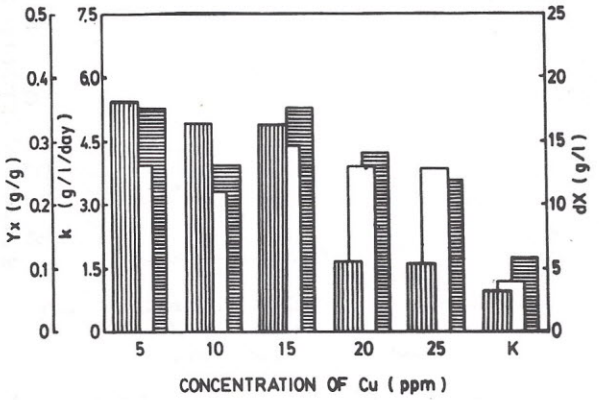

Figure 3. Growth efficiency of *A.niger* ATCC 11414 (4 days at 30°C) at various concentrations of Cu^{2+} ion, in glucose medium A. ▥ k, □ dX, ▤ Yx

K, control; k, initial linear-growth rate (g/l/day); dX, total biomass production (g/l); Y_X, biomass production per-unit glucose consumed (g/g).

The effects of copper were firstly investigated since it was toxic to the mould at higher concentrations [1]. Copper ion were studied at concentrations in the range of 5 - 25 ppm, using glucose medium A.

It is shown in Figure 2 and Figure 3, that the growth of *A.niger* ATCC 11414 decreased with the increase of copper concentrations. It is interesting to note that in the active growth period (0 - 2 days of incubation), the growth of this strain appeared to be linear rather than logarithmic as commonly shown by bacterial growth. This was possibly associated with the formation of pellets by *A.niger* ATCC 11414, which was also commonly observed during this study. Limitation of nutrient diffusion into the pellets resulted in growth restriction as had been reported by Rhigelato [14].

Figure 3 clearly indicates that in the range tested (5 - 25 ppm), copper ions were found optimum at the levels of 5 - 15 ppm for the mycelial growth of *A.niger* ATCC 11414, at 5% glucose. At concentrations higher than 15 ppm, copper ion lowered considerably the mycelial growth of this strain. The effect of copper ion at concentrations lower than 5 ppm was not tested.

Based on the results obtained, the influence of iron, zinc and manganese ions were then investigated using glucose medium B which contained 5 ppm Cu^{2+}. Each of these essential trace metals was tested at a fixed concentration of the others, i.e. 25 ppm for Fe^{2+}, 25 ppm for Zn^{2+}, and 25 ppb for Mn^{2+} ions.

Figure 4, Figure 5 and Figure 6 suggest that at concentrations of the trace metals in the range tested, the growth of *A.niger* ATCC 11414 slightly increased with the

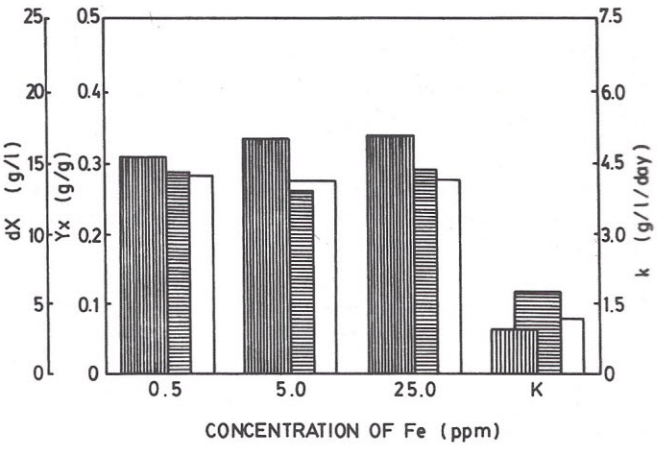

Figure 4. Growth efficiency of *A. niger* ATCC 11414 (4 days at 30°C) at various concentrations of Fe^{2+} ion, in glucose medium B which contained 5 ppm Cu^{2+}, 25 ppm Zn^{2+}, and 25 ppb Mn^{2+} ions.
▥ k, □ dX, ▤ Yx

k, initial linear-growth rate (g/l/day); dX, total biomass production (g/l); Y_X, biomass production per-unit glucose consumed (g/g); K, control culture (with no added trace metals).

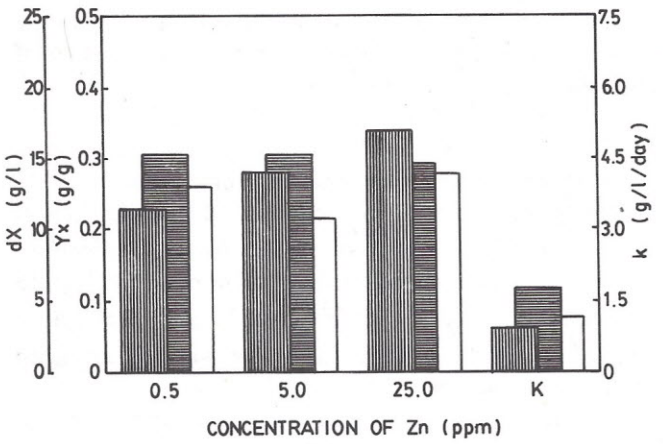

Figure 5. Growth efficiency of *A.niger* ATCC 11414 (4 days at 30°C) at various concentrations of Zn^{2+} ion, in glucose medium B which contained 5 ppm Cu^{2+}, 25 ppm Fe^{2+}, and 25 ppb Mn^{2+} ions.
▥ k, □ dX, ▤ Yx
k, initial linear-growth rate (g/l/day); dX, total biomass production (g/l); Y_X, biomass production per-unit glucose consumed (g/g); K, control culture (with no added trace metals).

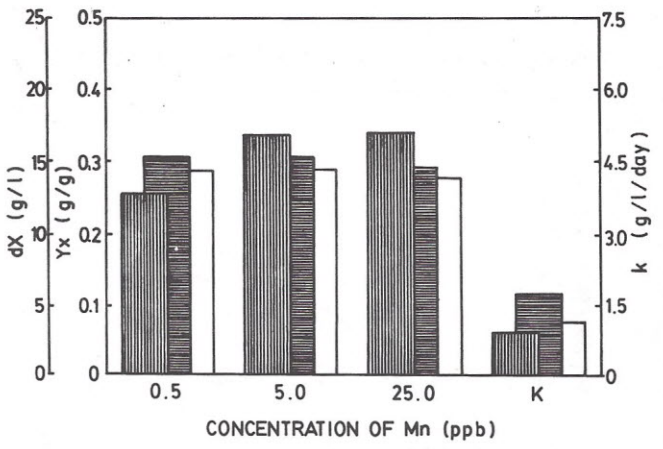

Figure 6. Growth efficiency of *A.niger* ATCC 11414 (4 days at 30°C) at various concentrations of Mn^{2+} ion, in glucose medium B which contained 5 ppm Cu^{2+}, 25 ppm Fe^{2+}, and 25 ppm Zn^{2+}.
▥ k, □ dX, ▤ Yx
k, initial linear-growth rate (g/l/day); dX, total biomass production (g/l); Y_X, biomass production per-unit glucose consumed (g/g); K, control culture (with no added trace metals)

increased Fe^{2+} (0.5 - 25 ppm), Zn^{2+} (0.5 - 25 ppm) and Mn^{2+} (0.5 - 25 ppb) concentrations. It was found that 5 - 25 ppm Fe^{2+} and 5 - 25 ppb Mn^{2+} were sufficient for the growth of *A.niger* ATCC 11414. The growth of the strain increased with the increase of Zn^{2+} (5 - 25 ppm). The levels of the above essential trace metals obtained in this study were higher than those quoted by Cochrane [1] for normal growth of fungi, *i.e.* 0.01 - 0.1 ppm copper, 0.1 - 0.3 ppm for iron, 0.5 - 1.0 ppm for zinc, and 5 - 10 ppb for manganese ions. Unfortunately, no data of the level of carbon source were reported so that rational comparisons could not be made. The optimum concentrations of the essential trace metals obtained in this study were also higher than those reported by Steinberg [9], *i.e.* copper 0.04 ppm, iron 0.20 ppm, zinc 0.18 ppm, and manganese 20 ppb. Differences in the organism, cultural conditions and level of the carbon source were possibly the factors causing the differences in the levels of these trace metals required.

In glucose medium B containing copper (25 ppm), iron (25 ppm), zinc (25 ppm) and manganese ions (25 ppb) *A.niger* ATCC 11414 gave biomass production of 13.9 g/l after 4 days at 30°C.

The ability of trace metals to form complex compounds with citric acid and other organic acids of the TCA cycle is an important aspect in studying the effects of essential trace metals on the growth of *A.niger*. This is mainly based on the fact that *A.niger* is a potential organism producing those acids.

The effect of ligand on the growth of *A.niger* ATCC 11414 was determined by stability constants of metal-ligand complex. Choudhary and Pirt [15,16] found that organic ligands with strong chelating abilities (ethylene-diamine-tetra acetic acid, diaminocyclohexane-N,N-tetra acetic acid and diethylenetriamine-penta acetic acid) had no effect on the mycelial growth of *A.niger*. The effect of pH on the stability constants of metal-ligand complex for various trace metals and ligands had been dealt with by Ringbom [17].

Effect of medium composition on the growth of *A.niger* ATCC 11414

Based on the results of the study on the effect of trace metals described in the previous section, glucose medium G of different compositions (Table 1) were tested as the growth medium for *A.niger* ATCC 11414. One of these glucose media tested (medium G4) had the same composition as that of Steinberg's medium, except that sucrose was replaced with glucose. Steinberg [8,9] obtained that the following medium was optimum for mycelial growth of *A.niger* Strain-W,: sucrose (50 g/l), NH_4NO_3 (2.06 g/l), KH_2PO_4 (0.55 g/l), $MgSO_4.7H_2O$ (0.22 g/l), Cu^{2+} (0.05 ppm), Fe^{2+} (0.30 ppm), Zn^{2+} (0.20 ppm), Mn^{2+} (25 ppb) and Mo^{6+} (20 ppb).

In this experiment, it was verified that the mycelial growth of *A.niger* ATCC 11414 would increase by the addition of molybdenum (20 ppb), and by increasing the NH_4NO_3 content of the medium (1.5 g/l) to a level as that of Steinberg's medium (2.06 g/l).

The mycelial growth of *A.niger* ATCC 11414 on glucose media G are presented in Figure 7 and Figure 8.

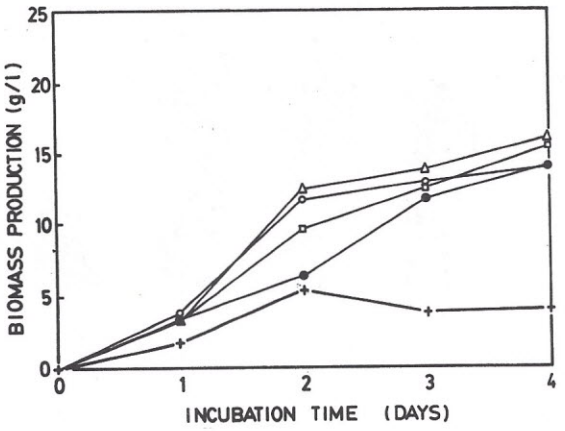

Figure 7. Growth of *A.niger* ATCC 11414 in glucose medium G of different compositions, as compared with that in medium glucose B.
○ Medium G1, Δ Medium G2, □ Medium G3,
● Medium G4, + Medium G5

The study demonstrated that in the active mycelial growth phase (0-2 days of incubation), the consumption of glucose was about 55 - 83 %, depending on the composition of the glucose medium.

It was found that *A.niger* ATCC 11414 gave biomass productions of 13.8 g/l, 16.1 g/l, 15.4 g/l, 14.0 g/l and 4.1 g/l after 4 days of incubation at 30°C, from the media G1, G2, G3, G4 and G5, respectively.

Figure 7 and Figure 8 clearly indicate that with the same strain and the same medium conditions (4 days at 30°C), medium G2 was found to be superior compared with either Steinberg's medium (medium G4) or with other glucose media tested.

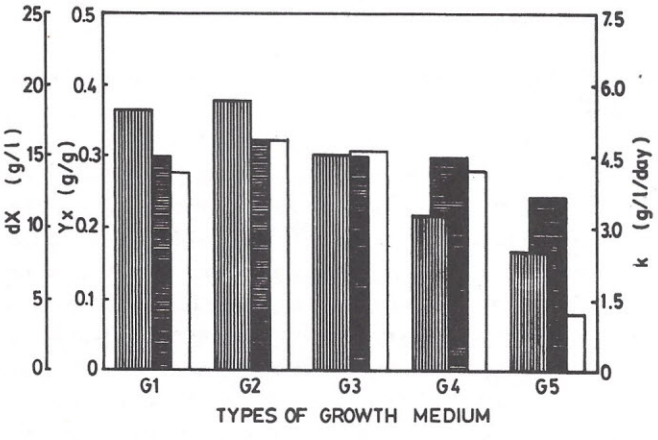

Figure 8. Growth efficiency of *A.niger* ATCC 11414 (4 days at 30°C) in glucose medium G, with different compositions.
▥ k, □ dX, ▤ Yx
k, initial linear-growth rate (g/l/day); dX, total biomass production (g/l); Y_X, biomass production per-unit glucose consumed (g/g).

It was concluded that medium G2 was optimal for the growth of *A.niger* 11414, with a biomass production of more than 16 g from 50 g glucose.

Comparing the biomass production of *A.niger* ATCC 11414 in glucose medium B (13.9 g/l) mentioned in the previous section with that obtained from the medium G1 (13.8 g/l), it is apparent that the increased NH_4NO_3 content (from 1.50 g/l to 2.06 g/l) gave no improvement in biomass production. While increasing the content of KH_2PO_4 (0.55 g/l to 1.20 g/l) and $MgSO_4.7H_2O$ (0.22 g/l to 0.50 g/l) in the medium G4 the biomass production was only increased slightly, i.e. from 14.0 g/l to 15.4 g/l.

On the other hand, the addition of molybdenum (20 ppb) resulted in the increase of biomass production from 13.8 g/l to 16.1 g/l. This confirmed the beneficial effect of this element for the growth of *A. niger*, which was reported by Steinberg [7]. Molybdenum, but neither gallium nor scandium, had been reported to have an effect on the growth of *A.niger* [6,9]. This positive effect of molybdenum was found to be associated with the role of this element in the reduction of nitrate ion and its intermediate reduction products in *A.niger*, so that the mould was able to utilize ammonium nitrate effectively as a nitrogen source.

Strains of *A.niger* which do not accumulate citric acid had been reported to have a high growth rate, and that highly acidogenic ones are relatively slow growing [1]. *A.niger* ATCC 11414 used in this study was identified as a citric acid producing strain [18]. According to Berry *et al.* [2], good citric acid producing strains usually form 10 - 15 g/l dry weight of mycelium, during the process of citric acid fermentation. It is indicated in Figure 9, that after 4 days of incubation at 30°C, *A.niger* ATCC 11414 produced 16.1 g/l biomass with a glucose consumption of 49.8 g/l. While in the glucose medium containing no added trace metals (Figure 1), the strain gave biomass production of only 3.90 g/l with a glucose consumption of 10.95 g/l, at the same cultivation conditions.

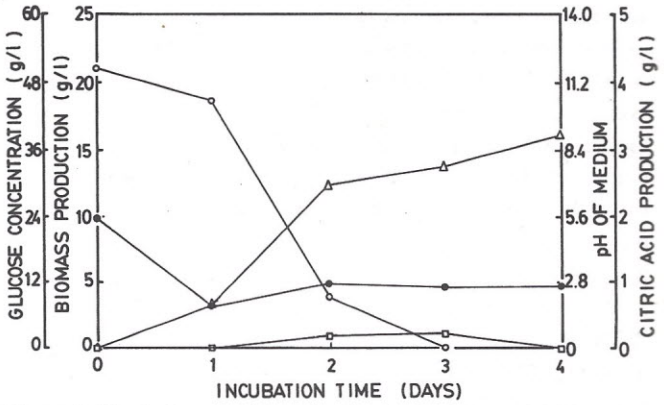

Figure 9. Typical growth and chemical changes during the cultivation of *A.niger* ATCC 11414 in a glucose medium G (medium G2).
○ Glucose concentration
□ Citric acid production
Δ Biomass production
● pH of medium

SERBA-SERBI IPTEK

GAMBARAN SEJARAH BIOTEKNOLOGI

Sejarah dari suatu teknologi terbukti merupakan sumber informasi penting untuk analisa ekonomi, dan adalah titik awal untuk memahami kekuatan, kelemahan, kepentingan dan tingkat dari teknologi tersebut.

Akar dari bioteknologi modern adalah fermentasi makanan dan minuman, suatu kegiatan industri yang sejak beberapa abad yang lalu telah dikenal oleh hampir seluruh masyarakat. Dasar dari proses fermentasi ini merupakan gabungan dari empirisme dan tradisi. Fermentasi alkohol dimulai paling sedikit pada masa Mesir kuno. Hingga sekarang alkohol hasil fermentasi ini baik dalam volume maupun harga jauh melebihi produk fermentasi baru seperti obat-obatan, asam-asam amino dan etanol hasil industri fermentasi. Terlepas dari efek racun etanol, titik jual yang esensial dari hasil-hasil fermentasi alkohol, masih belum diketahui sepenuhnya. Profil cita rasa dan sifat-sifat organoleptis dari bir, anggur, keju dan hasil-hasil lainnya masih belum dapat dijelaskan secara molekuler. Proses fermentasi ini masih mempunyai unsur pengikat yang kuat dari kepandaian khusus dan kebiasaan. Namun dengan beberapa penyederhanaan dan pengendalian yang cermat, industri fermentasi telah mencapai sukses yang nyata, misalnya pada industri anggur di Amerika.

Terlepas dari kesamaan dalam hal proses dan mikro-organisme, untuk pengertian ekonomi terdapat perbedaan cukup besar antara industri yang mengandalkan kepandaian khusus dan industri fermentasi pada pembuatan suatu senyawa kimia. Suatu perbandingan antara biaya produksi bir dan biaya produksi alkohol dalam industri menggambarkan hal ini dengan nyata. Etanol dijual seharga $ 10 - $ 20 per liter dalam keadaan tidak murni dalam bentuk larutan encer dari minuman beralkohol. Penghasil etanol industri harus puas dengan harga $ 0,45 - $ 1,0 per liter untuk etanol 95 % atau absolut, yang mungkin menyebabkan orang bertanya-tanya apakah industrialis ini melakukan bisnis yang keliru. Terlepas dari umur dan kesetiaan pada metoda tradisional, industri fermentasi minuman beralkohol yang bersifat menuntut keakhlian khusus ini telah menunjukkan pertumbuhan yang sejajar atau bahkan melebihi pertumbuhan ekonomi secara keseluruhan masyarakat yang telah maju. Karena keistimewaan cita rasa dan sifat toksik yang khas dari minuman beralkohol ini, maka proses ini tidak mempunyai saingan dari proses-proses lain.

Sebaliknya produksi bahan kimia curah secara fermentasi seperti etanol telah didikte oleh biaya, terutama yang berkaitan dengan industri petrokimia. Industri ini berkembang sebagai akibat pertumbuhan pesat dari fermentasi tradisional pada akhir abad-19 dan awal abad-20, untuk memasok senyawa-senyawa pada industri kimia dan industri senjata yang bermunculan. Dalam masa-masa perang senyawa yang mula-mula dihasilkan adalah etanol, gliserol, aseton dan butanol, diikuti asam-asam organik seperti asam sitrat, laktat, fumarat dan glukonat. Kecuali asam glukonat, fermentasi asam-asam organik memperoleh keuntungan, namun amatlah tergantung pada tersedianya molases sebagai hasil samping dari industri pemurnian gula, yang perdagangannya berkembang ke seluruh dunia. Perkembangan teknologi fermentasi yang pada umumnya berdasarkan pada fermentasi tangki aduk, tidak mendapat cukup perhatian dibandingkan dengan pengembangan ilmu kimia, sehingga industri fermentasi mengalami stagnasi. Pada masa pasca perang dunia ke-2 pada saat industri petrokimia berkembang dengan amat pesat, teknologi fermentasi mengalami kemerosotan yang cukup berarti. Beberapa proses, misalnya produksi asam sitrat untuk makanan, dapat bertahan namun keperluan industri hampir seluruhnya dipasok dari hasil sintesa kimiawi, kecuali bila diatur oleh pemerintah.

Bersambung ke hal. 52

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