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Nutritional Studies on the Growth of the Rapamycin-Producing *Streptomyces hygroscopicus*

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Abstract During our previous studies on the relationship between nutrient requirements of *S. hygroscopicus* C9 and rapamycin biosynthesis, we developed chemically-defined media containing among other nutrients, aspartic acid, arginine, histidine, or ammonium sulfate. However, these media ("Cheng *et al.* medium" and "Lee *et al.* medium") showed very slow growth characterized by a very long lag phase of growth. In an attempt to develop a chemically-defined or semi-defined medium to support more rapid growth and increased cell production, we have carried out studies to shorten the lag phase. Of the various additives tested, vitamin-free casein acid hydrolysate was the most significant by shortening the lag phase by 2–3 days. Mixtures of amino acids failed to replace casein acid hydrolysate. The active principle passed through an ultrafilter with a molecular weight cutoff of 1,000 and thus may be a peptide. The present work has yielded a semi-defined medium which should be useful for further growth studies on *S. hygroscopicus* C9.

Key words: Casein acid hydrolysate, rapamycin, *Streptomyces hygroscopicus*

Rapamycin (sirolimus) is produced by the bacterium *Streptomyces hygroscopicus*, which was originally isolated from a soil sample from Easter Island [8]. The molecule contains a large macrolide ring with a heterocyclic moiety (which is derived from free pipecolic acid, which in turn comes from lysine) [7]. Since its discovery as an antifungal agent, rapamycin has been found to possess antitumor and immunosuppressant properties. Immunosuppressants are of great use in medicine for organ transplantation. These compounds decrease the immune system's response to new organs, thus improving the chances that the foreign organ will be accepted by the body. Organ allografting methods have employed the immunosuppressants cyclosporin A, FK506 (=tacrolimus, which is structurally similar to rapamycin) and, more recently, rapamycin. Rapamycin has great use in medicine because it is more efficacious and less toxic than the other two compounds [9].

In the past, only little was done on the nitrogen nutritional requirements of *S. hygroscopicus* C9. A medium containing the complex ingredient, yeast extract, was used by Paiva *et al.* [6]. This medium supported rapid growth, but the use of a complex medium makes it difficult to determine the relationship between nutrition of *S. hygroscopicus* and rapamycin biosynthesis. The problem is that yeast extract contains hundreds of compounds and it is not known which ones the bacterium takes up. This problem can be avoided by the use of a chemically defined medium, which contains pure chemical nutrients, or simplified by a semi-defined medium which contains, for example, a vitamin-free protein hydrolysate. Utilization of these nutrients can be easily studied after an ideal medium is devised.

A medium that contains a combination of three amino acids (L-arginine, L-histidine, L-aspartic acid) as the nitrogen sources for growth, plus L-lysine as a precursor of rapamycin, was devised by Cheng *et al.* [1, 2]. Growth in this medium is very slow with a long growth lag of several days. Another medium that contains only one amino acid, L-lysine, plus ammonium sulfate as the nitrogen source for growth, is the Lee *et al.* medium [5]. This medium also suffers from many days of growth lag. It should be noted that L-lysine, a precursor of rapamycin, is not used for growth. The present study describes our progress in devising a semi-chemically defined medium which supports a much shorter growth lag and high cell production.

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MATERIALS AND METHODS

Microorganism

Streptomyces hygroscopicus C9, a spontaneous mutant derived from strain AY-B1206 [7], was used in the studies. A spore suspension was prepared as described in our previous paper [4].

Seed and Fermentation Media

Seed medium consisted of (g/l) starch 10, peptone 6.0, yeast extract 6.0, Casamino acids (Difco, Franklin Lakes, NJ, U.S.A.) 1.5, $MgSO_4 \cdot 7H_2O$ 0.5, and K_2HPO_2 1.0. Davis-Wong chemically defined fermentation medium consisted of (g/l) 2-(N-morpholino)ethanesulfonic acid monohydrate (MES) buffer 21.3, fructose 20, mannose 5.0, L-arginine 2.0, L-histidine·HCl 1.0, L-lysine 5.0, $(NH_4)_2SO_4$ 5.3, K_2HPO_4 0.87, NaCl 5.0, and (mg/l) $ZnSO_4 \cdot 7H_2O$ 60.0, $MgSO_4 \cdot 7H_2O$ 2.56, $MnSO_4 \cdot H_2O$ 12.0, $FeSO_4 \cdot 7H_2O$ 100, $(NH_4)_6MO_7O_{24} \cdot 4H_2O$ 18.0, $Na_2B_4O_7 \cdot 10H_2O$ 10.0, $CoCl_2 \cdot 6H_2O$ 10.0, $CuCl_2 \cdot 2H_2O$ 1.3. The pH of the fermentation medium was adjusted to 6.0 with 9 M potassium hydroxide. The sugars, fructose and mannose, were autoclaved separately from the rest of the medium and added to the medium prior to inoculation.

Seed and Fermentation Cultures

One ml of spore suspension for inoculum was added to 25 ml of seed medium in a 250-ml baffled flask and the flask was shaken at 220 rpm, 30°C for 48 h. The seed culture was centrifuged at 8,000 ×g, 4°C for 25 min and the collected cells were washed with 25 ml 0.1 M MES buffer (pH 6.0). The washed suspension was centrifuged to collect cells at 8,000 ×g, 4°C for 25 min. The cells were suspended in 10 or 20 ml 0.1 M MES buffer (pH 6.0). One ml of washed cells was added to 25 ml fermentation medium in 250-ml baffled flasks. The inoculated flasks were incubated at 220 rpm, 30°C and the samples were taken from flasks daily. Dry cell weight was determined as described in our previous paper [4].

Ultrafiltration of Casein Acid Hydrolysate

Casein acid hydrolysate (ICN, Costa Mesa, CA, U.S.A.) (750 mg) was dissolved in 6 ml water and filtered through cellulose filter YM1, which has a molecular weight cutoff (MWCO) of 1,000, with Ultrafiltration Cell Model M-3 (Millipore, Bedford, MA, U.S.A.). Filtrate and retentate, equivalent to 3 g/l casein acid hydrolysate, were added into the Davis-Wong medium prior to inoculation.

RESULTS AND DISCUSSION

Early Studies on Media of Cheng *et al.* and Lee *et al.*

Early studies on modifications of the Cheng *et al.* [1, 2] medium and the Lee *et al.* [5] medium aimed at combining the important components of these media and eliminating unnecessary components. Lysine concentration was decreased in the Cheng *et al.* medium from 10 g/l to 5 g/l for reasons of economy; the change did not decrease growth or rapamycin production. Next, the importance of the other three amino acids of the Cheng *et al.* medium was studied in the simpler Lee *et al.* medium. Aspartic acid was found to have no positive effect and was eliminated. The other two amino acids, L-arginine and L-histidine were found to stimulate growth and their concentrations were optimized. The improved medium was the Cheng *et al.* medium lacking aspartic acid and containing L-lysine at 5 g/l, L-arginine at 2 g/l, L-histidine·HCl at 1 g/l, and ammonium sulfate at 5.3 g/l. This was called the Davis-Wong chemically defined medium and used as the basis for further studies. It was also found in these early studies that the seed medium was improved by use of soluble starch instead of the previously used glucose.

Additives to the Davis-Wong Medium

The Davis-Wong medium was an improvement over the earlier chemically defined medium but still was characterized by a long lag phase. Additives that failed to improve the growth pattern were soybean oil, corn oil, cottonseed oil, α-cyclodextrin, *p*-aminobenzoic acid, and thiamine. The following additives did reduce the growth lag: salt-free acid hydrolysate of casein, enzyme hydrolysate of casein, yeast extract, and peptone. The most active additive was the casein acid hydrolysate. A mixture of 14 amino acids other than lysine, aspartic acid, arginine, and histidine did reduce growth lag somewhat, but did not approach the activity of casein acid hydrolysate. Many other combinations of amino acids were tested, but none could replace casein acid hydrolysate.

Studies on Casein Acid Hydrolysate

Using the Davis-Wong medium as the basal medium, further studies have been done on the effect of casein acid

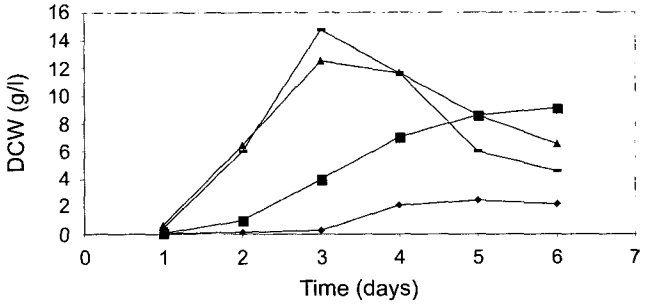

Fig. 1. Effect of concentration of casein acid hydrolysate on the growth of *Streptomyces hygroscopicus* C9 in Davis-Wong fermentation medium.

The control medium (◆) and the control medium plus 1 (■), 5 (▲), or 10 g/l (—) vitamin-free casein acid hydrolysate were used.

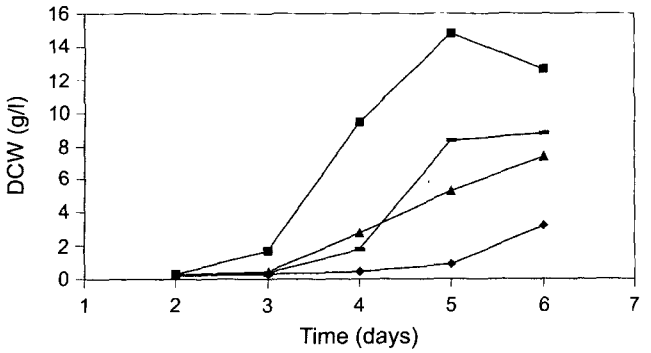

Fig. 2. Effect of casein acid hydrolysates from different manufacturers at 3 g/l on the growth of *Streptomyces hygroscopicus* C9 in Davis-Wong fermentation medium.
The control medium (◆) and the control plus casein acid hydrolysate media from different manufacturers, ICN (■), Gibco (▲), or Difco (—), were used.

hydrolysate on the growth of *S. hygroscopicus*. Different concentrations (1, 5, 10 g/l) of casein acid hydrolysate were added and the growth performance was measured as dry cell weight. The lowest concentration shortened the lag phase by 1 d. The higher concentrations of casein acid hydrolysate (5, 10 g/l) had an even greater effect, shortening the lag phase by 2 d, increasing growth rate and the extent of growth (Fig. 1).

Casein acid hydrolysates from different manufacturers, i.e., ICN, Difco, and Gibco (Carlsbad, CA, U.S.A.), were tested as shown in Fig. 2. Although they all shortened the lag phase, ICN vitamin-free casein acid hydrolysate showed the greatest effect.

Vitamin-free casein acid hydrolysate (ICN) was dissolved and filtered through an ultrafilter of 1,000 MWCO. Both the filtrate and retentate were tested for their effect on growth of *S. hygroscopicus* C9. Figure 3 shows that the filtrate was more active than the retentate and almost

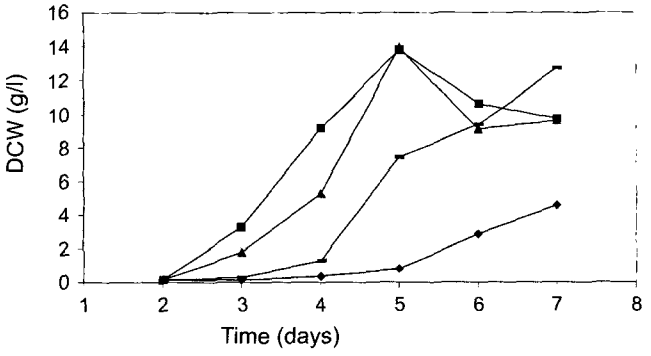

Fig. 3. Effect of filtrate and retentate obtained by ultrafiltration of ICN vitamin-free casein acid hydrolysate with a 1,000 MWCO filter.
The amounts tested were equivalent to the 3 g/l of casein acid hydrolysate. The control medium (◆) and the control medium plus ICN casein acid hydrolysate (■), filtrate (▲), or retentate (—) were used.

Table 1. Composition of a newly developed semi-defined medium.

Component	Amount per liter
Casein acid hydrolysate (ICN)	3.0 g
Fructose[a]	20 g
Mannose[a]	5.0 g
L-Arginine	2.0 g
L-Histidine·HCl	1.0 g
L-Lysine	5.0 g
$(NH_4)_2SO_4$	5.3 g
K_2HPO_4	0.87 g
NaCl	5.0 g
$ZnSO_4 \cdot 7H_2O$	60.0 mg
$MgSO_4 \cdot 7H_2O$	2.56 mg
$MnSO_4 \cdot H_2O$	12.0 mg
$FeSO_4 \cdot 7H_2O$	100 mg
$(NH_4)_6MO_7O_{24} \cdot 4H_2O$	18.0 mg
$Na_2B_4O_7 \cdot 10H_2O$	10.0 mg
$CoCl_2 \cdot 6H_2O$	10.0 mg
$CuCl_2 \cdot 2H_2O$	1.30 mg
MES buffer	21.3 g
pH (9 M KOH)	6.0

[a]Autoclaved separately.

reproduced the effect of the casein acid hydrolysate. This indicates that the nutrient required to shorten the lag phase is a relatively small molecule, possibly a peptide. The residual activity of the retentate could be due to larger peptides containing the active peptide as part of their structures.

Our results indicate that rapid and extensive growth of the rapamycin producer, *S. hygroscopicus* C9 requires some nutrient in vitamin-free casein acid hydrolysate. We have not been able to replace the factor with combinations of amino acids or with sources of fatty acids which sometimes contaminate casein acid hydrolysates [3]. The active molecule is small since it can pass through a 1,000 MWCO ultrafilter. At this point, it appears that it is a peptide but further work will be needed to identify it. Rapamycin production in the semi-defined medium developed in this work (Table 1) is low due to the presence of phenylalanine and methionine in the casein hydrolysates. These amino acids inhibit rapamycin formation [1]. Thus, the small growth-stimulatory peptide will have to be identified before a chemically-defined medium can be developed for use in genetic and physiological studies on rapamycin production.

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