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Carbon source nutrition of rapamycin biosynthesis in *Streptomyces hygroscopicus*

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SUMMARY

Chemically-defined media were developed for rapamycin production by *Streptomyces hygroscopicus*. Thirty-five carbon sources were tested for their effect on production. Eight failed to support growth and seven appeared to repress or inhibit rapamycin formation. The best combination of two carbon sources were 2% fructose and 0.5% mannose. Acetate and propionate, which are known to contribute most of the carbon atoms of the lactone ring, were unsatisfactory for growth and/or rapamycin production.

INTRODUCTION

Rapamycin is a potent immunosuppressant which also has antifungal and antitumor activities [4]. It was discovered as an antifungal antibiotic produced by *Streptomyces hygroscopicus* strain AY B994 which was isolated from a soil sample collected on Easter Island [8,13]. Rapamycin is an unusual nitrogen-containing triene macrolide with a very large 31-membered lactone ring [3,11]. Precursors of its biosynthesis are acetate, propionate, methionine, pipecolate and shikimate [5,6,7]. Its total chemical synthesis was accomplished in 1993 [1].

Since the immunosuppressive potency of rapamycin is somewhat greater than FK 506 [12], a related non-polyene macrolide, and 150 times greater than cyclosporin A [2], a cyclic peptide consisting of 11 amino acids, and its toxicity is less than cyclosporin [10], this agent is of great current clinical interest. There is a paucity of published information on the rapamycin fermentation [8,9] and its control. Nothing has been published on the nutrition of the producing organism. In this paper, we report on the carbon source nutrition of *S. hygroscopicus* with respect to rapamycin production.

This paper is dedicated to Professor Herman Jan Phaff in honor of his 50 years of active research which still continues.

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MATERIALS AND METHODS

Microorganisms

The rapamycin-producing microorganism, *Streptomyces hygroscopicus* strain AY-B1206 obtained from the Ayerst Research Laboratories, Montreal, Canada (now Wyeth-Ayerst Research, Princeton, NJ, USA) and a higher-producing variant C9 isolated by us, were used. The two strains were allowed to sporulate on a medium consisting of (g L^{-1}): glucose 1.0, yeast extract 1.0, malt extract 2.5, agar 20, pH 7.0. The cultures were grown in petri dishes at 28 °C for 10 days. Three milliliters of 20% glycerol were added to each dish. The resulting spore suspension was stored at −20 °C.

Inoculum

A seed culture was initiated by adding 0.2 ml of the thawed spore suspension to a 250-ml baffled Erlenmeyer flask containing 40 ml of medium consisting of (g L^{-1}): soluble starch 10.0, peptone 6.0, yeast extract 6.0, casamino acids 1.5, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 1.0, pH 7.0. Incubation was at 28 °C for 40 h on a rotary shaker operating at 200 r.p.m. The harvested seed culture was centrifuged at 4 °C for 20 min and the cells were suspended in 100 mM MES (pH 6.0) buffer. One milliliter of this suspension with a dry cell weight (DCW) of 4 mg was used as inoculum per flask of chemically-defined production media.

Fermentation

Two chemically-defined media were developed in this work as reported in the Results and Discussion section. Their compositions are described in Table 1. Production cultures were prepared in duplicate 250-ml baffled flasks containing 25 ml of medium, inoculated, and incubated for 8 days at 28 °C on a rotary shaker operating at 200 r.p.m.

TABLE 1

Composition of two media

Component	Medium 1 (per liter)	Medium 2 (per liter)
Glycerol	20.0 g	—
αD(+)Glucose*	10.0 g	—
D-Fructose*	—	20.0 g
D(+)Mannose*	—	5.0 g
Na-L-aspartate	1.5 g	1.5 g
L-Arginine	0.5 g	0.5 g
L-Histidine·HCl	0.5 g	0.5 g
K_2HPO_4	2.0 g	2.0 g
KH_2PO_4	2.0 g	2.0 g
NaCl	5.0 g	5.0 g
$ZnSO_4·7H_2O$	60 mg	60 mg
$MgSO_4·7H_2O$	256 mg	256 mg
$MgCl_2·6H_2O$	510 mg	510 mg
$MnSO_4·H_2O$	12 mg	12 mg
$FeSO_4·7H_2O$	100 mg	100 mg
$(NH_4)_6Mo_7O_{24}·4H_2O$	18 mg	18 mg
$Na_2B_4O_7·10H_2O$	10 mg	10 mg
$CoCl_2·6H_2O$	10 mg	10 mg
$CuCl_2·2H_2O$	1.3 mg	1.3 mg
Na_2SO_4	360 mg	360 mg
MES buffer	21.3 g	21.3 g
pH	6.0	6.0

* Autoclaved separately.

Extraction

One milliliter of fermented whole broth was centrifuged for 5 min. The supernatant fluid was transferred by pipette into a test tube and the pellet was extracted twice, each time by shaking with 1 ml of methanol for 20 min at room temperature. The pooled extracts were added to the supernatant and assayed.

Determinations

All data shown represent the average of duplicate flasks. Sampling was done on days 6 and 8. The data presented represent the maximum of the two samplings.

We determined dry cell weight (DCW) by adding 1 ml of whole broth to 1 ml of distilled water and 3 ml of 3 N HCl. The suspension was homogenized by ultrasonic treatment for 90 s on a Branson Sonifier (Cell Disruptor 200) (Branson Ultrasonics Corp., Danbury, CT, USA) and its turbidity was measured using the Klett Summerson Photoelectric Colorimeter (Klett Manufacturing Co. Inc., New York, NY, USA) with a red filter. Dilutions were made until the Klett readings were in the range between 10 and 100 Klett units. A broth DCW of 1 mg ml^{-1} was found to be equivalent to 286 Klett units.

Bioassay of rapamycin was performed on the pooled supernatant fluid plus extracts described above by paper disc-agar diffusion using *Candida albicans* ATCC 11651 as the assay microorganism. The assay medium consisted of (g L^{-1}) peptone 2 g, glucose 5 g, agar 10 g, pH 6.0 and was seeded with *C. albicans* before pouring of plates. The plates were incubated overnight at 37 °C. Volumetric production refers to mg per liter of whole fermentation broth.

RESULTS AND DISCUSSION

Preliminary studies on development of a chemically-defined medium

Our earlier studies on incorporation of labelled precursors into rapamycin were done in a complex medium containing yeast extract [5,6,7]. Elimination of the yeast extract markedly reduced growth and rapamycin production. However before starting nutritional experiments on rapamycin production, we wanted to develop a chemically-defined medium which could support growth. To begin this effort, we omitted yeast extract and amino acids from the medium described by Paiva et al. [5]. It was composed of 2% glucose (autoclaved separately) and mineral salts as follows (per liter): 2 g K_2HPO_4, 2 g K_2HPO_4, 5 g NaCl, 1.5 g $CaCO_3$, 60 mg $ZnSO_4·7H_2O$, 256 mg $MgSO_4·7H_2O$, 510 mg $MgCl_2·6H_2O$, 12 mg $MnSO_4·H_2O$, 100 mg $FeSO_4·7H_2O$, 18 mg $(NH_4)_6Mo_7O_{24}·4H_2O$, 10 mg $Na_2B_4O_7·10H_2O$, 10 mg $CoCl_2·6H_2O$, 1.3 mg $CuCl_2·2H_2O$, 360 mg Na_2SO_4, pH 6.0. This basal medium contained no nitrogen source.

Over 20 individual amino acids were tested at 2 g L^{-1} as sole nitrogen source. Growth was estimated visually due to extreme pellet formation which made optical measurements difficult. The best growth was obtained with threonine, alanine, arginine, asparagine, histidine, aspartic acid and ornithine. In a second test, each of these amino acids was tested at concentrations of 0.5–4 g L^{-1}. Aspartic acid was found to be best. Using aspartic acid at 1.5 g L^{-1}, we next screened the amino acids again individually as secondary and tertiary amino acid sources at 0.5 g L^{-1}. Arginine and histidine were best and further experiments showed that a combination of 1.5 g L^{-1} L-aspartic acid, 0.5 g L^{-1} L-arginine and 0.5 g L^{-1} L-histidine·HCl provided a good nitrogen source mixture for growth of *S. hygroscopicus*. A screening of carbohydrates showed that a mixture of 10 g L^{-1} glucose and 20 g L^{-1} glycerol supported good growth. Observation of growth was facilitated by substitution of the insoluble $CaCO_3$ with 100 mM MES buffer. As a result of these preliminary studies, chemically-defined medium 1 was developed. Its composition is given in Materials and Methods.

Effect of carbon sources on the formation of rapamycin

In a series of four experiments, 35 carbon sources were examined, including polysaccharides, oligosaccharides, monosaccharides, and organic acids as well as lipid materials. All were tested at 30 g L^{-1} in chemically-defined medium 1 as replacements for glucose plus glycerol. Twenty carbon sources supported growth and rapamycin production (Table 2). The best carbon sources for rapamycin production by *S. hygroscopicus* AY-B1206 were D-fructose, D(+)mannose, D(+)galactose, *i*-inositol, D-mannitol, D(+)xylose and D(+)cellobiose. Eight carbon sources which failed to support growth were dextrin, inulin, salicin, L(−)sorbose, D-sorbitol, ethanol, lactic acid and sodium acetate. Seven carbon sources appeared to exert catabolite repression or inhibition, i.e. they

TABLE 2

Growth and rapamycin production with 20 carbon sources

Carbon source (30 g L^{-1})	Growth DCW (g L^{-1})	Rapamycin vol. production (% of control)*	Rapamycin specific production (% of control)**
D-Fructose	1.4	169	172
i-Inositol	1.7	195	103
D(+)Cellobiose	2.0	174	131
D(+)Galactose	0.5	137	412
D-Mannitol	1.3	130	90
D(+)Mannose	0.6	119	298
D(+)Xylose	0.6	106	265
Soluble starch	2.8	89	45
L(+)Arabinose	1.4	65	69
meso-Erythritol	0.8	64	72
α D(+)Glucose	1.4	61	66
Glycerol	1.7	59	109
α D(+)Melibiose	0.3	53	263
D(+)Maltose	0.6	50	125
D-Ribose	1.1	43	60
D(+)Raffinose	2.2	38	26
Xylan	—	33	—
Laminarin	1.8	31	24
α L-Rhamnose	0.2	30	228
Pectin	1.2	21	19

* Controls (2% glycerol + 1% glucose) were 20, 17, 26 and 18 mg L^{-1} of whole fermentation broth in the four experiments.
** Controls were 13, 11, 29 and 13 mg g^{-1} DCW in the four experiments. Strain AY-B1206 was used for these experiments.

TABLE 3

Effect of secondary carbon sources on rapamycin production

Additional carbon source* (10 g L^{-1})	Growth DCW (g L^{-1})	Rapamycin vol. production (mg L^{-1})	Rapamycin specific production (mg g^{-1} DCW)
None	1.1	24	22
D(+)Mannose	1.2	57	48
D(+)Glucose	1.2	39	33
D-Mannitol	1.3	38	29
D(+)Maltose	1.1	38	34
D(+)Xylose	1.3	37	28
Methyloleate	3.2	37	12
i-Inositol	1.2	33	28
Glycerol	1.1	31	29
D(+)Galactose	1.2	28	23
Soluble starch	1.3	28	21
α L-Rhamnose	1.7	25	16
D(+)Raffinose	1.1	23	21

* The basal medium contained 20 g L^{-1} fructose.
Strain C-9 was used in this experiment.

supported growth but not rapamycin production. They were sucrose, α-D-lactose, D(+)trehalose, xylitol, propanol, methyloleate and oleate. Although most of the carbon atoms of the lactone ring of rapamycin are derived from acetate and propionate, neither of these is a satisfactory carbon source for the fermentation.

Effect of fructose concentration on the biosynthesis of rapamycin

One of the best carbon sources for rapamycin production on a volumetric as well as a specific basis was fructose (Table 2). It was tested at different concentrations with the higher-producing strain C9. Fructose at 20 g L^{-1} was found to be best for production of rapamycin. Higher concentrations showed no further effect.

Effect of carbon sources as secondary carbon source

Most secondary metabolite fermentations are improved when a secondary carbon source is employed in a chemically-defined medium. Using 20 g L^{-1} fructose, many of the carbon sources were tested as a secondary carbon source at 10 g L^{-1}. Table 3 shows that mannose was the best secondary carbon source. Also of interest in this experiment was the ability of methyloleate, which was considered to inhibit or repress rapamycin formation when tested as a sole carbon source, to markedly decrease the specific production of rapamycin. Further experimentation revealed that 5 g L^{-1} mannose was sufficient to give optimum production. Higher concentrations (up to 30 g L^{-1}) were not inhibitory but yielded no more rapamycin. The final chemically-defined medium resulting from this study is medium 2 which is being used to further examine nutritional regulation of rapamycin biosynthesis.

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