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Production and chemical characterization of antifungal metabolites from *Micromonospora* sp. M39 isolated from mangrove rhizosphere soil

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Summary

Micromonospora sp. M39 was selected as a producer of antifungal substances. Cell mass and antifungal activity of the organism was dependent on the carbon and nitrogen sources used. A combination of glucose, starch and glycerol as carbon sources at 1% (w/v) and corn steep powder as a nitrogen source at 0.25% (w/v) concentration in basal medium gave a maximum growth of 17.2 PCV/50 ml and *in vitro* an antifungal activity of 19.0 mm against the rice blast pathogen *Pyricularia oryzae* MPO 293. In the selected production medium, optimum antifungal activity of the crude extract of the strain M39 was on day 16 of fermentation at 28 ± 2 °C. The crude extract of the strain M39 was analyzed and characterized by HPLC-DAD-UV–visible spectra. The peaks with a retention time of 4.88 min had a u.v. spectrum identical to that of 2,3-dihydroxybenzoic acid. The peak with a retention time of 6.08 min was confirmed by an automated spectral library search as phenylacetic acid. Two other peaks in the chromatogram were identified as cervinomycin A1 with a retention time at 12.78 min and cervinomycin A2 with a retention time at 13.46 min.

Introduction

Actinomycetes with the highest recorded chemical diversity produce many secondary metabolites of novel structure and metabolites possessing a broad range of biological activities. As the frequency of novel bioactive compounds discovered from terrestrial actinomycetes decreases with time, industrial programmes are increasingly screening actinomycetes from diverse environments for their ability to generate new metabolites. Studies have shown that actinomycetes isolated from the marine environment are metabolically active and have adapted to life in the sea (Jensen *et al.* 1991). The requirements for discovery and isolation of new lead structures from natural sources are undisputed. This goal may be reached by investigating new microbial sources for production of bioactive compounds, such as non-*Streptomyces* actinomycetes. The genus *Micromonospora* Orskov 1923 is attracting wide attention as a new and important source of antifungal, antibacterial and antitumour agents (Mc Brien *et al.* 1995).

Microorganisms differ in the ways they respond to fermentation conditions and once the conditions are favourable they may produce a vast array of bioactive compounds. Tanaka (1992) demonstrated that the requirements for successful production of antifungal substances were (i) unique microorganism, (ii) specific fermentation conditions and (iii) sensitive methods for detection of activity. An identification system based on reversed phase gradient HPLC and computerized diode-array detection could be used for determination of the chemical diversity in crude extracts from microorganisms (Fiedler 1993).

Seventy *Micromonospora* strains were isolated from the rhizospheres and the roots of tropical mangrove trees (*Sonneratia* and *Rhizophora* sp.) collected from four mangrove stands of West Malaysia (Ismet *et al.* 2002). Among the strains, *Micromonospora* sp. M39 demonstrated strong antagonistic activity to a number of phytopathogenic fungi, bacteria and KB tumour cell line *in vitro* (Ismet 2003). Further, *Micromonospora* sp. M39 was found to produce secondary metabolites that induced mycelial swelling and lysis in *Pyr. oryzae* and *Ganoderma boninense* (Ismet 2003). The aims of this study were (a) to optimize the carbon and nitrogen sources for growth and production of antifungal substances by *Micromonospora*

sp. M39 and (b) to tentatively identify the components in the crude extract of the strain M39.

Materials and methods

Test strain

Micromonospora sp. M39 was isolated from mangrove rhizosphere soil (Ismet *et al*. 2002). The strain M39 in 30% glycerol (v/v) stock cultures was inoculated onto micromonospora agar medium (MMS). The MMS medium contained (g l^{-1}): yeast extract, 4.0; $CaCO_3$, 1.0; soluble starch, 20.0; glucose, 15.0; K_2HPO_4, 0.1; $MgSO_4$, 0.1; casein (N-Z amine A), 5.0; (artificial seawater) instant ocean, 17.0; agar, 18.0 in distilled water. The pH was adjusted to 7.0. The inoculated plates were incubated at 28 ± 2 °C for 2 weeks.

Inoculum preparation

Hundred millilitre of tryptic soy broth (TSB) (Sigma Co.) (pH 7.0) containing 0.3% (w/v) yeast extract was dispensed into each of several 250 ml Erlenmeyer flasks. The flasks were then autoclaved at 121 °C at 15 psi for 15 min. The well-grown culture of M39 on MMS agar was harvested carefully with a loop and suspended in 10 ml of sterile distilled water in a McCartney bottle. Then, each flask was inoculated with 5 ml culture suspension of M39. All the flasks were incubated on a rotary shaker incubator (Environ-Shaker 3597-1PR) at 150 rev min^{-1} at 28 ± 2 °C for 2 weeks. The culture broth was centrifuged at 5000 rev min^{-1} for 10 min at 25 ± 2 °C. The pellet of M39 was washed twice by resuspension in sterile distilled water and resuspended in 10 ml of sterile distilled water. Two millilitre of this suspension was used as inoculum in the carbon and nitrogen utilization studies.

Effect of varying carbon sources on growth and production of antifungal substances of M39

A basal medium contained peptone (w/v): 0.5%; yeast extract, 0.2%; $CaCO_3$, 0.3%; instant ocean, 1.7% and distilled water. The pH of the medium was adjusted to 7.3. Glucose, glycerol and starch, alone or in combination as carbon sources, were incorporated in the basal medium.

Fifty millilitre of the basal medium was dispensed in 250 ml Erlenmeyer flasks. Each carbon source at 1% concentration (w/v) was added to the medium and sterilized. The flasks were then inoculated aseptically with 2 ml of culture suspension of the M39. The control flasks contained 50 ml basal medium without the inoculum. Triplicate flasks, set up for each carbon source tested, were then incubated on a rotary shaker incubator (Environ-Shaker 3597-1PR) at 150 rev min^{-1} at 28 ± 2 °C for 18 days. The culture broth was centrifuged at 5000 rev min^{-1} for 10 min at 25 ± 2 °C and the packed cell volume (PCV/50 ml) was measured. The pH of the supernatant was recorded.

Effect of various nitrogen sources on growth and production of antifungal substances by M39

A basal medium contained (w/v): glucose 1%; glycerol, 1%; starch, 1%; $CaCO_3$, 0.3% and 1.7% instant ocean in distilled water. The pH was adjusted to 7.3. Corn steep powder (CSP) (Sigma Co.), soybean flour (SBF) (Sigma Co.) and cottonseed flour (CSF) (Sigma Co.) at 0.25% (w/v) concentration were used as the nitrogen containing sources. The culture conditions were as described above for optimization of carbon sources.

Effect of time on growth and production of antifungal compounds of M39

The constituents of production medium was selected from above studies. The medium consisted of (w/v): starch, 1.0%; glucose, 1.0%; glycerol, 1.0%; corn steep powder, 0.25%; peptone, 0.5%; yeast extract, 0.2%; $CaCO_3$, 0.3%; instant ocean 1.7% and distilled water. The pH of the medium was adjusted to 7.3. The time course fermentation study as described above was carried out for 18 days.

Crude extract preparation

The freeze-dried whole culture broth of each carbon and nitrogen source tested as well as from the time course study was used for the crude extract preparation. The freeze-dried sample was steeped in 100 ml of dichloromethane and methanol (v/v; 1:1) for 24 h at 28 ± 2 °C. The soaked sample was then sonicated at 42 KH_2 for 30 min and filtered through Whatman No. 1. The filtrate was evaporated at 50 °C to obtain the crude extract (Ismet 2003).

Antifungal activity in crude extract

The phytopathogenic fungus *Pyr. oryzae* was maintained on potato dextrose agar (PDA) (Difco Co.) plates at 28 ± 2 °C for 4 days. Antifungal activity using different crude extracts of freeze-dried whole fermentation broth of *Micromonospora* sp. M39 was tested using 6.0 mm paper discs impregnated with 20 μl (containing 0.6 mg/disc) of crude extract (Pisano *et al*. 1986). Standard antifungal discs containing 50 μg of cycloheximide/ml were used as the positive control, while paper-discs soaked in 20 μl methanol were used as the negative control. Triplicate plates set up for each of the crude extract of *Micromonospora* sp. M39 tested and incubated in the dark at 28 ± 2 °C for 5 days. After incubation, the diameter of the growth inhibition zone (mm) of *Pyr. oryzae*, if any, was recorded.

HPLC–UV–visible diode array detection (DAD) of metabolites in crude extract

The crude extract of the *Micromonospora* sp. M39 was analyzed by HPLC using the diode-array detection

method. The extract was dissolved in methanol (MeOH) to give a concentration of 1 mg ml^{-1} and 10 μl was injected into the HPLC column. The u.v. spectra of the crude extract of the strain M39 were compared with the retention times of u.v. spectral data from known antibiotics and other metabolites in natural products libraries, to predict the natural product class or make a tentative identification of the compound (Fiedler 1993).

Statistical analysis

Data reported are as means of triplicate values of pH, growth and activity for each carbon and nitrogen source studied and the time course study. Analysis of variance (ANOVA) on changes of pH, growth and activity data was conducted. Tukey test ($P < 0.05$) was used for overall comparisons of the results. The statistical Package SPSS (Statistical Package for Social Science) version 9.0 was used.

Results and discussion

Effect of varying carbon sources on growth and antifungal substance production by Micromonospora *sp. M39*

The effects of different carbon sources on growth, pH and production of antifungal substances of the *Micromonospora* sp. M39 are shown in Table 1. A combination of glucose, starch and glycerol at 1% (w/v) concentration of each carbon source in a basal medium gave the highest packed cell volume of 12.5 and an antifungal activity of 21.3 mm when compared to single carbon sources tested (Table 1). The antifungal activity increased about 4.0-fold when glucose, glycerol and starch were present in the basal medium compared to the activity with starch alone as the carbon source. During the initial fermentation phase, glucose may be used as a rapid growth enhancer or energy stimulator for M39. However, as fermentation progressed, the glucose level can be reduced and a slowly utilized carbon source such as starch and glycerol can substitute as carbon sources for the growth and production of bioactive substances. It was observed by Huck *et al.* (1991) that glucose was metabolized and consumed during the early phases of fermentation while during the middle phases of growth, as glucose levels reduced, starch may be used as the carbon source.

The basal medium containing soluble starch or glycerol as sole carbon sources demonstrated poor growth and low activity of the crude extract of strain M39 against *Pyr. oryzae* when compared to when glucose was a sole source of carbon (Table 1). This finding did not correlate with other studies. For example, soluble starch and glycerol had been used as the superior carbon sources for the production of antibacterial antibiotic, juvenimicin from *Mic. chalcea* var. *izumensis* in a chemically defined fermentation broth (Hatano *et al.* 1976).

However, in this study, glucose when used alone increased both PCV and antifungal activity when compared to glycerol or starch (Table 1). Glucose is generally a suitable carbon source for growth of microorganisms. However, studies have shown that glucose interferes with the biosynthesis of many antibiotics and it has not been frequently used for the growth and production of bioactive compounds from certain actinomycetes (Huck *et al.* 1991). In selected cases, polysaccharides or oligosaccharides have been found to be better carbon sources than glucose.

Different carbon sources had a significant effect on the pH in the fermentation media ($F = 6.87$, $P = 0.001$). In the basal medium they also had a significant effect on biomass produced ($F = 8.86$, $P = 0.001$) and on antifungal activity ($F = 15.78$, $P = 0.001$) of M39.

Effect of various nitrogen sources on growth and antifungal substance production by M39

The effects of different organic nitrogen sources on growth, pH and production of antifungal substance are shown in Table 2. The highest antifungal activity was

Table 1. Effect of various carbon sources on growth and antifungal activity of the *Micromonospora* sp. M39 against *Pyricularia oryzae* MPO 293[a].

Carbon sources	Media pH after fermentation	Packed cell volume (PCV/50 ml)	Antifungal activity (zone, mm)	Activity ratio[b]
Basal media (BM)	7.0 ± 0.3	2.9 ± 0.06	0.0	0.0
BM + starch [c]	8.2 ± 0.6	4.0 ± 0.9	6.07 ± 0.6	1
BM + glucose	7.9 ± 0.05	8.1 ± 1.2	14.03 ± 1.8	2.3
BM + glycerol	8.0 ± 0.2	6.9 ± 0.8	10.0 ± 0.6	1.7
BM + glycerol + glucose	6.0 ± 0.0	10.8 ± 1.6	15.0 ± 1.5	2.5
BM + starch + glycerol	7.7 ± 0.2	6.9 ± 0.7	11.0 ± 3.06	1.8
BM + glucose + glycerol + starch	6.9 ± 0.6	12.5 ± 1.9	21.3 ± 2.2	3.5

[a] Packed cell volume (PCV/50 ml), pH and activity (mm) against *Pyr. oryzae* were determined at 18 days of incubation at 28 ± 2 °C; Means are with standard error (SE).
[b] Activity ratio (AR) = Antifungal activity of crude extract with different carbon sources/antifungal activity of crude extract with starch soluble.
[c] Soluble starch (lowest activity) was considered as control and activity compared with other carbon source.

observed in the basal medium supplemented with corn steep powder (CSP) at 0.25% (w/v) concentration (Table 2). Satoi *et al.* (1980) reported that corn steep liquor had been used for the production of mycinamycins from *Mic. griseorubida*. In this study, the antifungal activity increased 2.0-fold when corn steep powder was added to the basal medium compared to that of the basal medium alone.

Increase of antifungal substance production was observed to be dependent on the nitrogen sources used in the basal medium (Table 2). This was corroborated by the findings of Huck *et al.* (1991) for other actinomycetes. There was also evidence that a wide range of complex natural raw materials such as corn steep liquor, yeast extract, soybean flour, etc. may be used as nitrogen source for the bulk production of antibiotic from *Micromonospora* and other actinomycetes.

The effects of different nitrogen sources on pH was not significant ($F = 1.87$, $P = 0.21$) while the growth of M39 was also not significantly different for the different nitrogen sources used in the basal medium ($F = 1.27$, $P = 0.35$). However, antifungal activity of the crude extract with different nitrogen sources varied significantly ($F = 10.97$, $P = 0.003$).

Selection of production medium

A production medium consisting of glucose, 1.0%; glycerol, 1.0%; starch, 1.0%; corn steep powder, 0.25%; peptone, 0.5%; yeast extract, 0.2%; $CaCO_3$, 0.3% and instant ocean, 1.7% in distilled water (pH 7.3) was selected based on the high antifungal activity against *Pyr. oryzae*. It was evident that glycerol, glucose and starch (0.1–2.0%) as carbon sources and corn steep liquor (0.3–1.0%) as a nitrogen source in the fermentation medium were suitable for the production of bioactive substances from actinomycetes (Huck *et al.* 1991).

Tanaka (1992) reported that the addition of carbon source at 0–5% and a nitrogen source at 0–3% concentrations in the media enhanced the production of antibiotic substances. Further, complex nutrients such as starch, glucose, glycerol, yeast extract, peptone, corn steep liquor, have been used for the production of antifungal, antibacterial and antitumour compounds from *Micromonospora* species by many researchers. Examples include: (a) novel macrolide antibiotics mycinamycins were produced by *Mic. griseorubida* sp. nov. in a production medium containing glucose as the sole carbon source and corn steep liquor as the sole nitrogen source and (b) dapiramicin, a highly effective antibiotic against *Rhizoctonia solani* (sheath blight of rice pathogen) was produced by *Micromonospora* sp. SF-1917 in a complex production medium containing soluble starch, glucose, peptone, yeast extract, soybean meal and $CaCO_3$ (Shomura *et al.* 1983).

In basal medium, in addition to carbon and nitrogen sources, other components such as yeast extract, peptone, $CaCO_3$ and instant ocean were used and these might have influenced the growth and production of bioactive compounds, too. There is evidence that yeast extract and peptone are effective as nitrogen sources to maintain growth in the fermentation media. Yeast extract has also been reported as a disperser of mycelium in the fermentation broth of many actinomycetes (Kobinata *et al.* 1993). Further, studies have indicated that for the effective and selective fermentation for bioactive substances from actinomycetes, a combination of carbon, starch, glycerol and glucose can be used with other complex nutrients, buffering agents and salts (Huck *et al.* 1991).

The pH values within the growth-permissible range of the strain had an effect on antibiotic production. The pH of the media may be controlled by the addition of calcium carbonate ($CaCO_3$) in the fermentation media. Calcium carbonate is one of the most common buffering agents used to avoid excessive acidic conditions. In the fermentation broth of strain M39, $CaCO_3$ a buffering agent maintained the pH and variation was minimal.

Nutritional ingredients in a fermentation medium are indispensable for the growth and production of bioactive substances of microorganisms. Tanaka *et al.* (1986) observed that in certain cases some excessive nutritional components such as glucose, amino acids and other carbon and nitrogen sources affected antibiotic production in fermentation broth. However, the reverse may not always be true. For example, the production of 16-membered macrolide antibiotic tylosin by *Str. fradiae* was not affected by 4% glucose added to a starch-based production medium (Tanaka *et al.* 1986).

Table 2. Effect of different nitrogen sources on growth and antifungal activity of the *Micromonospora* sp. M39 against *Pyr. oryzae* MPO 293[a].

Nitrogen sources	pH of the media after fermentation	Packed cell volume (PCV/50 ml)	Antifungal activity (zone of inhibition in mm)	Activity ratio[b]
1. Basal[c] medium (BM)	7.9 ± 0.5	8.9 ± 1.1	11.0 ± 0.6	1.0
2. BM + soybean flour	7.7 ± 0.3	7.9 ± 0.7	13.0 ± 2.3	1.18
3. BM + corn steep powder	6.8 ± 0.2	11.4 ± 2.2	24.0 ± 2.0	2.18
4. BM + cotton seed flour	7.6 ± 0.3	9.9 ± 0.6	15.0 ± 1.5	1.36

[a] Packed cell volume (PCV/50 ml), pH and antifungal activity were determined after 18 days of incubation; Means in the column are mentioned with standard error (SE).
[b] Activity ratio (AR) = Antifungal activity of crude extract of different nitrogen source/antifungal activity of crude extract of basal medium.
[c] Basal medium was considered as control and activity compared with other nitrogen sources.

Time course study on the optimum production of antifungal substance of Micromonospora *sp. M39*

The effect of different incubation times on the production of antifungal substances of the strain M39 are shown in Figure 1. It was observed that the production of antifungal substances increased with the increase of cell growth of M39. According to Hoskisson *et al.* (2001) this may reflect the fact that antibiotic production is spatially constrained to mycelial forms of the organism. Further, the production of bioactive substances by M39 started at 6 days of fermentation and maximum antifungal activity was observed on day 16 of fermentation period.

Examples are (a) antibacterial and antitumour depsipeptide antibiotic thiocoraline production from a marine *Micromonospora* sp. L-13-ACM2-092 was maximum after 96 h of fermentation (Romero *et al.* 1997) and (b) in the fermentation broth of *Mic. echinospora* gentamicin was first detected after 72 h, peaking after 120 h and rapidly declined at 144 h (Hoskisson *et al.* 2001). However, there are no known reports that maximum antifungal activity was achieved at 16 days of fermentation period. From these results it is concluded that M39 was a very slow growing *Micromonospora* strain and thus needed a long fermentation time for the bioactive substance production compared to other producer *Micromonospora* spp. Further studies should be carried-out to improve the growth rate of the *Micromonospora* strain in fermentation broth. Physiological factors such as temperature, pH, aeration and oxygen tension need to be studied during the fermentation of the M39. It is possible that these factors may influence the production.

These findings were not corroborated by other studies done. Though the biomass was increasing after 16 days of incubation, the antifungal activity gradually decreased. This observation is contrary to the concept that increasing biomass gives higher bioactivity. However, this observation was not further investigated in this study.

The effect of incubation time on pH change during growth of M39 in the fermentation broth was significant ($F = 5.34$, $P = 0.001$). The incubation time also had a significant effect on the growth of the strain M39 ($F = 16.51$, $P = 0.001$) and the antifungal activity in the culture broth ($F = 25.90$, $P = 0.001$).

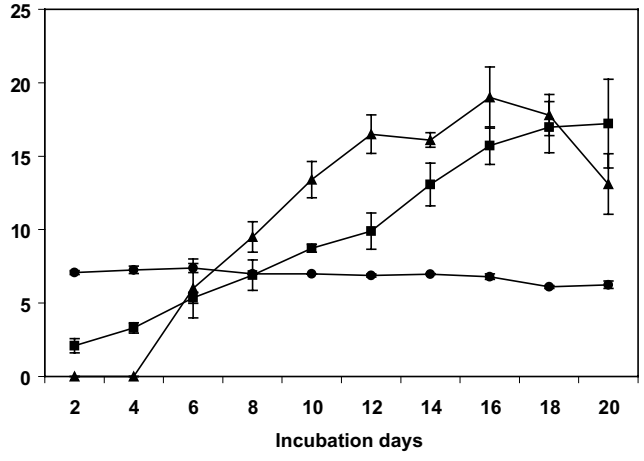

Figure 1. Time course study on growth and production of antifungal substances by the *Micromonospora* sp. M39. Bar represents standard error of the mean (incubation temperature: 28 ± 2 °C, inoculum size: 2 ml/50 ml). (–●–) Media pH, (–■–) PCV/50 ml, (–▲–) Inhibition zone (mm).

Identification of metabolites in crude extract of Micromonospora *sp. M39 using HPLC–UV–visible spectral data*

A series of chromatographic peaks were recorded in the HPLC elution profile of the crude extract of M39. The peaks with a retention time of 4.88 min had a u.v. spectrum identical to 2,3-dihydroxybenzoic acid. Benzoic acid is an antifungal and antibacterial agent that is used as a preservative of foods. Recently, it was demonstrated that two bacteriostatic agents, enterocin and wailupemycin, both polyketides were derived from benzoic acid produced by a marine streptomycete, *Str. maritimus* (Hertweck & Moore 2000). According to Hertweck & Moore (2000) benzoic acid is also a common metabolite in eukaryotic systems and is a component of many important natural products, including salicylic acid, cocaine, taxol and the zaragozic acids. However, this is the first report that *Micromonospora* species produced 2,3-dihydroxybenzoic acid as a antifungal and antibacterial metabolite in fermentation broth.

The peak with a retention time of 6.08 min was confirmed by the automated spectral library search as phenylacetic acid. Phenylacetic acid reported as a antifungal compound is produced by many microorganisms. *Str. humidus* produced phenylacetic acid, which was active against fungi (Hwang *et al.* 2001). There are, however, no available reports that *Micromonospora* species too, produced phenylacetic acid as an antifungal metabolite in fermentation broth.

Two other peaks in the chromatogram were identified as cervinomycin A1, with a retention time at 12.78 min and cervinomycin A2, with a retention time at 13.46 min. Cervinomycins A1 and A2 are new xanthone antibiotics, produced by *Str. cervinus* Takahashi and Omura sp. nov. AM-5344T (Nakagawa *et al.* 1987). They are highly active against Gram-positive bacteria, including *Staphylococcus aureus*, some anaerobes (*Clostridium*, *Peptococcus* and *Bacteroides*) and mycoplasmas (Nakagawa *et al.* 1987). A novel antitumour compound calicheamicin is the only representative of a xanthone structure antibiotic produced by a member of the genus *Micromonospora* (Lee *et al.* 1987). To our knowledge this is the first report that *Micromonospora* species produce cervinomycins A1 and A2.

A mixture of compounds in a complex fermentation broth of *Micromonospora* sp. M39 were detected with

HPLC–u.v. spectra. Nelson *et al.* (1986) reported that *Mic. purpureochromogenes* subsp. *halotolerans* produced a complex of antibiotics with five components, which were detected using HPLC–u.v. visible spectra. There are several reports that single *Micromonospora* species may produce a mixture of compounds in a complex fermentation medium (Takenaka *et al.* 1998). For example, juvenimicin was active against *Bacillus subtilis*, *B. cereus* and *Sta. aureus* and was found to be composed of eight components, which were produced in a complex medium (Hatano *et al.* 1976). Fiedler (1993) has reported that the HPLC screening using u.v. visible absorbance spectral libraries did not replace other methods of structure elucidation. It permits, however, at a very early stage of investigation, judgement on peaks of an HPLC elution profile regarding identity or association with a certain class of natural substances.

Conclusions

Antifungal substance production is dependent on the carbon, nitrogen source and incubation time used. A combination of starch, glucose and glycerol at 1% concentration as carbon sources and corn steep powder at 0.25% concentration as nitrogen source in basal medium enhanced the antifungal activity of M39. *Micromonospora* sp. M39 produced a number of substances such as 2,3-dihydroxybenzoic acid, phenylacetic acid and cervinomycins in complex fermentation medium.

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