Isolation of endophyic bacteria from purwoceng (*Pimpinella alpina* Kds.)

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Abstrak

Latar belakang: *Purwoceng (Pimpinella alpina Kds.) merupakan tanaman obat langka yang berkhasiat sebagai afrodisiak. Tanaman yang mengandung kumarin tersebut berinteraksi dengan bakteri endofit. Senyawa kumarin dalam industri dimanfaatkan untuk bahan aditif makanan dan parfum. Penelitian ini bertujuan untuk mendapatkan bakteri endofit dari tanaman purwoceng, menganalisis kemampuan tumbuh bakteri endofit dalam medium yang mengandung kumarin, dan pengaruh bakteri tersebut terhadap jumlah kumarin dalam medium pertumbuhan.*

Metode: *Isolasi bakteri endofit dilakukan secara langsung dari akar dan daun purwoceng. Seleksi bakteri endofit penghasil kumarin dilakukan berdasarkan kemampuan bertahan hidup pada medium cair ammonium salt sugar (ASS) yang mengandung infusa herba purwoceng. Pengaruh bakteri terhadap jumlah kumarin di dalam medium pertumbuhan diuji melalui percobaan kultivasi isolat bakteri terpilih pada medium yang sama. Jumlah kumarin dalam kultur dideteksi menggunakan teknik kromatografi lapis tipis (KLT).*

Hasil: *Sembilan isolat bakteri endofit yang berhasil diisolasi dari akar dan daun tanaman purwoceng mampu bertahan hidup pada medium basal yang diberi infusa herba purwoceng dengan waktu generasi (g) 2,7-5,07 jam dan kecepatan pertumbuhan spesifik (μ) 0,14-0,26/jam. Kultivasi isolat terpilih menunjukkan bahwa BAP_5 menghasilkan senyawa dengan Rf 0,27 yang diduga sebagai turunan kumarin. Bakteri BAP_5 mampu tumbuh dengan jumlah kumarin 1072 arbitrary unit (AU) dalam medium.*

Kesimpulan: *Bakteri endofit dapat diisolasi dari tanaman purwoceng dan secara in vitro mampu mempertahankan jumlah kumarin yang terkandung di dalam medium.* ***(Health Science Indones 2012;1:31-6)***

Kata kunci: *bakteri endofit, purwoceng, Pimpinella alpina Kds, kumarin*

Abstract

Background: Purwoceng (*Pimpinella alpina* Kds.) is a medicinal plant species used as aphrodisiac. Like any other plants, the coumarin containing plant probably interacts with endophytic bacteria. Coumarin and its derivatives has wide biological activity spectrum as antifungal, anticoagulation, anti inflamation and it can be an additive in certain food or cosmetic additive. This study aimed to isolate endophytic bacteria from purwoceng, to assess the growth of endophytic bacteria within coumarin containing medium and to reveal the affect of endophytic bacteria to the coumarin content of the medium.

Methods: Endophytic bacteria were isolated from purwoceng roots and leaves. Pure culture of endophytic bacteria was selected by growing the bacteria in the ammonium salt sugar medium containing purwoceng herbal infusion. The effect of the bacteria to coumarin content in the medium was assessed through the cultivation of chosen bacteria in medium that was similar with the medium used in the selection step. Coumarin content in the medium was detected by using thin layer chromatography (TLC).

Results: Nine isolates obtained from purwoceng roots and leaves could be alive in the basic medium containing purwoceng herbal infusion and had generation time (g) 2.7-5.7 hours and specific growth rate (μ) 0,14-0,26/hour. Cultivation of chosen isolate showed that BAP_5 could grow in the medium containing 1072 arbitrary unit (AU) of coumarin. The TLC exhibited *Rf* 0.27 of the compound that was assumed as coumarin.

Conclusion: Endophytic bacteria were successfully isolated from purwoceng and prevented the coumarin loss from the medium. ***(Health Science Indones 2012;1:31-6)***

Key words: endophytic bacteria, purwoceng, *Pimpinella alpina* Kds, coumarin

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Purwoceng (*Pimpinella alpina* Kds.) is a medicinal plant used as aphrodisiac, diuretic and tonic. The plant belongs to Apiaceae or Umbelliferae and contains derivatives of coumarin such as bergapten, marmesin, 4-hydroxycoumarin, umbelliferon and psoralen.[1] Coumarin and its derivatives has wide biological activity spectrum as antifungal, anticoagulation, anti inflamation and it can be an additive in certain food or cosmetic additive.[2-5]

In the wild purwoceng grows on very specific agroclimatic environment and it has historical ethnobotany that it can be a source of endophytic bacteria isolate.[6] Endophytic bacteria reside within the tissue of roots, stem and leaves, and some of them are able to produce or involved in production of plant host secondary metabolite.[7] So far study on purwoceng is focused on cultivation, *in vitro* culture, phytochemical and pharmacology, therefore study of endophytic bacteria from purwoceng is needed to complete other related studies.[8] The aims of this study were to isolate endophytic bacteria from purwoceng, to assess the growth of endophytic bacteria within coumarin containing medium and to reveal the effect of endophytic bacteria to the coumarin content of the medium.

METHODS

Organism. Purwoceng (*Pimpinella alpina* Kds.) plant was taken from Sikunang village at Dieng plateu, Central Java; bacteria were isolated from purwocengs roots and leaves.

Isolation and purification of endophytic bacteria. Purwocengs roots and leaves were washed in tap water then soaked in 5.3% sodium hypochloride solution for 3 minutes, followed by soaking them in the 70% ethanol for 3 minutes. After that, the roots and leaves were rinsed twice using sterile aquadest. The last rinsing water inoculated into nutrient agar (NA) (Difco) to confirm the surface sterilization process. Sterilized sample was wiped by using sterile tissue paper. One gram sample was pounded in the sterile pestle. While pounding, phosphate buffer, half strength nutrient broth (NB) (Difco) and sterile aquadest were added. The concentrated liquid was diluted with sterile aquadest until the concentration of 10^{-3}. Then, the aliquot was inoculated on the NA medium used *spread plate* and *pour plate* method, and incubated in room temperature for 24-72 hours.[9] Colonies grew separately and looked different transferred onto the NA medium. Several reinoculation was carried out until the colony morphologivally pure.

Bacteria selection. Fourty eigth hour inoculum inoculated into culture flask containing 90 ml ASS (ammonium salt sugar) medium that was added with 12 g purwoceng powder for each liter medium. The growth of bacteria was observed by counting the colony forming unit (CFU) and measuring the absorbance of culture liquid by using spectrophotometer (Shimadzu 1240) at λ 600 nm. Isolate that exhibited the shortest generation time and the highest specific growth rate was chosen for bacterial cultivation.

Recorded absorbance value at the logaritmic phase was used to make bacteria growth curve by calculating the growth kinetics. This kinetics formula correlates growth rate Constanta (k), generation time (g) and specific growth rate (μ).[10]

Cultivation. The 48 hour inoculum inoculated into the same medium as used in the selection step. The batch culture of endophytic bacteria then incubated for 96 hours at 28^{o} C. After the incubation period the medium was extracted and qualitatively analyzed by using thin layer chromatography.

Thin Layer Chromatography. The 96 hour batch culture was refined by using Whatman No. 1 filter paper. Moreover, the filtrate was added with chloroform, shook and let it separate become two layers after a moment. The upon layer, water fraction, was poured gently into porcelain cup and the lower layer, chloroform fraction, was poured into another porcelain cup, then both of them were dried.

Extract of chloroform fraction was diluted in 5 ml chloroform then spotted onto siliga gel 60 F254 (Merck). Coumarin standard (Sigma) in the form of white crystal was dissolved in the chloroform and spotted. Eluent used were benzene: glacial acetic acid: water = 20:5:1.[11] Compounds separated by TLC on silica gel plate were observed under the λ 254 and 366 nm UV light. TLC Scanner (Camag 3) was used for chromatogram profile making.

RESULTS

Isolation and purification of endophytic bacteria. Isolation of bacteria from roots and leaves of purwoceng

showed that there were 24,000-672,000 cells of bacteria in each gram sample (Table 1). The purification step resulted in 9 morphologically different colonies and bacillus, Gram positive were predominant. (Table 2).

Bacteria selection. The growth kinetics of nine isolate bacteria grown in ASS medium was added with purwoceng herbal infusion is shown in Table 3. BAP_5 isolate was the bacteria showing the shortest generation time (2.7 hours) and the highest specific growth (0.26/hour).

Thin Layer Chromatography (TLC). The TLC result revealed that standard coumarin compound exhibited the *Rf* 0.27. Quantitative analysis of TLC result showed that the coumarin content detected in the ASS medium that had been added with purwoceng herbal infusion changed after 96 hour incubation. In the uninoculated medium the coumarin content decreased 447,4 arbitrary unit (AU) after 96 hour incubation and in the BAP_5 bacteria culture it only decreased 31,1 AU (Figure 1 and 2).

Table 1. Number of endophytic bacteria within purwocengs roots and leaves tissue.

Isolation method	Sample	Number of bacteria CFU 10^3/ml	Number of bacteria per 1 gram of sample (cell)
Spread	Leaves	16	96.000
	Roots	112	672.000
Pour	Leaves	4	24.000
	Roots	20	120.000

Table 2. Colony and cell morphology of endophytic bacteria

Isolate	Colony Morphology	Cell morphology
BAS_2	curled, cream, smooth, urbanite, undulate	Bacillus, Gram positive
BAS_4	curled, cream, finely granular, urbanite, undulate	Bacillus, Gram positive
BAS_{52}	myeloid, cream, undulate, pollinate	Bacillus, Gram positive
BAP_3	myeloid, cream, smooth, urbanite, fimbriate	Bacillus, Gram positive
BAP_5	myceloid, cream, coarsely granular, raised with concave beveled edge, undulate	Bacillus, Gram positive
BDS2	round, cream, smooth, effuse, undulate	Bacillus, Gram positive
BDS_3	myceloid, cream, coarsely granular, raised, fimbriate	Bacillus, Gram positive
BDP_1	round, yellow, finely granular, pulvinate, entire	Coccus, Gram positive
BDP_5	round, white, smooth, convex, entire	Bacillus, Gram positive

Table 3. Growth kinetics of endophytic bacteria in the ASS medium added with purwoceng herbal infusion.

Isolate	Generation time (g) (hour)	Specific growth rate (μ)($hour^{-1}$)
BAS_2	3.05	0.23
BAS_4	5.07	0.14
BAS_{52}	2.99	0.23
BAP_3	4.32	0.16
BAP_5	2.70	0.26
BDS_2	3.43	0.20
BDS_3	3.25	0.21
BDP_1	4.37	0.16
BDP_5	3.35	0.21

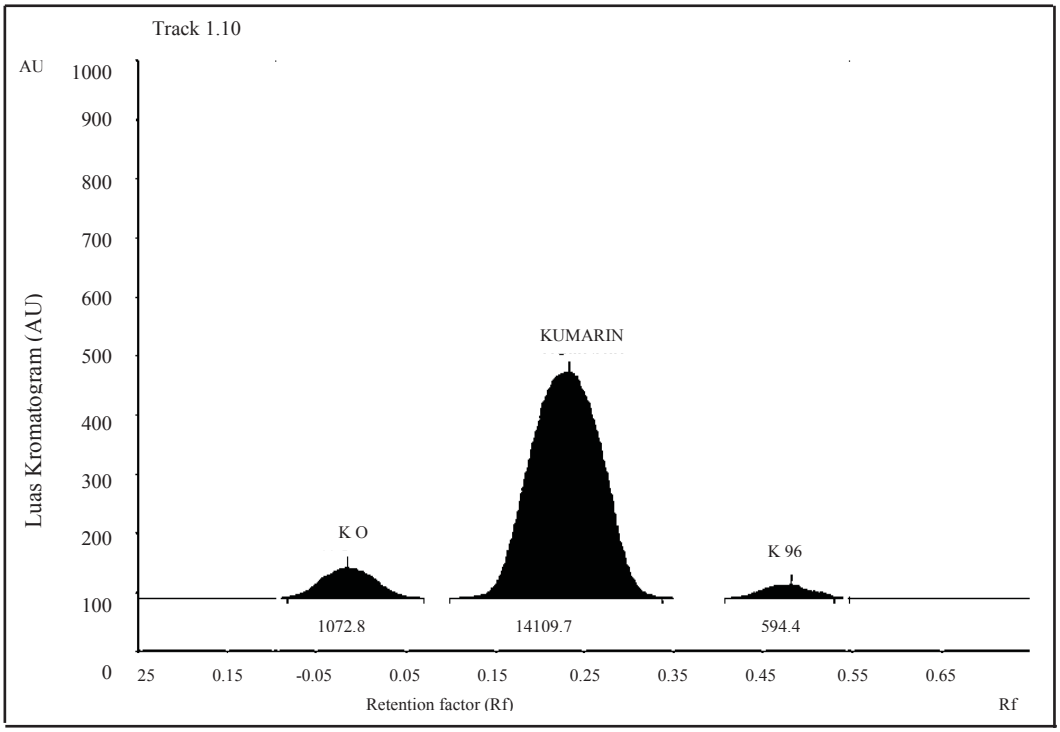

Figure 1. Coumarin content in the uninoculated ASS medium added with purwoceng herbal infusion. K0: 0 hour incubation; K96: 96 hour incubation.

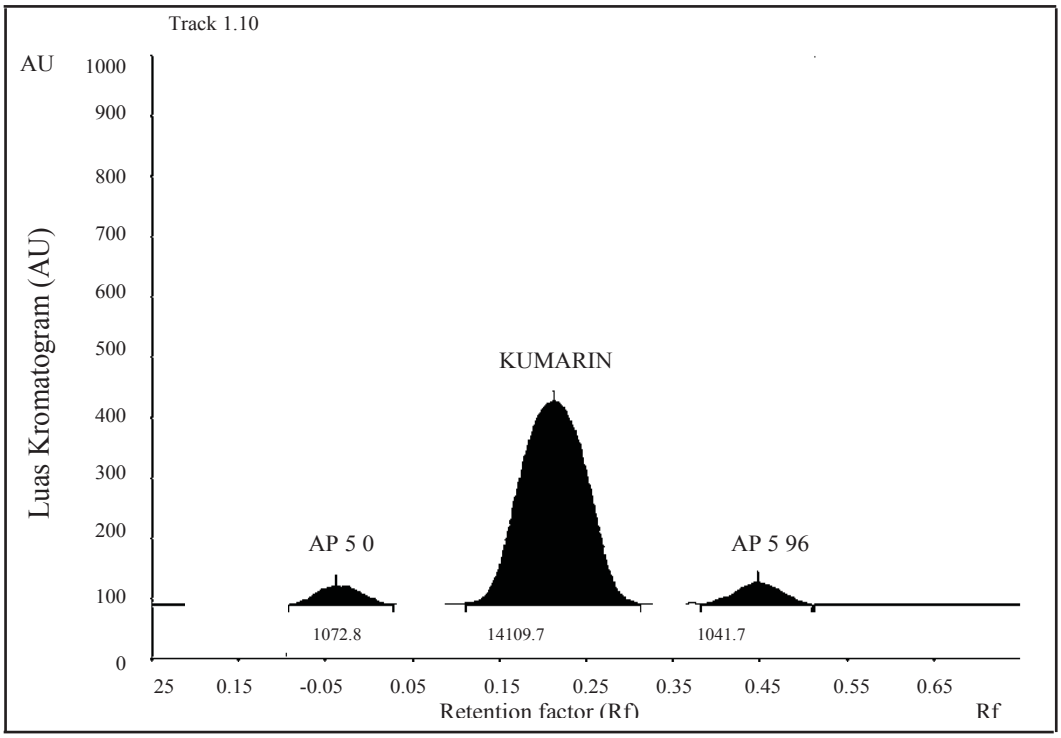

Figure 2. Coumarin content in the batch culture of BAP5 endophytic bacteria with the ASS medium added with purwoceng herbal infusion.
AP50: 0 zero hour incubation; AP596: 96 hour incubation

DISCUSSION

The number of endophytic bacteria isolated from roots of purwoceng seems to be higher than the number of bacteria isolated from leaves. This result is similar with a study showing that in most of plant, roots is resided by more endophytic bacteria than stem, leaves, fruit, tuber and ovulum.[12] The number of endophytic bacteria in the plant tissue is various depends on the plant organ resided in. The endophytic bacteria strain is having specific correlation with the plant organ resided in as well. The specification related to the phytochemical content in each plant organ.[7]

The higher number of endophytic bacteria found in the roots presumably due to more various chemical content of roots than the chemical content found in other organs. Phytochemical compound of purwoceng roots consists of alcaloid, flavonoid, coumarin, triterpenoid, saponin and tannin, whereas flavonoid and tannin cannot be found in the leaves.[13]

The growth of endophytic bacteria was assessed by growing the bacteria within ASS minimum medium added with purwoceng herbal infusion as the carbon source for the bacteria. The minimum medium gives the bacteria macro element such as N, H, S, O, P, K and Mg and the purwoceng infusion provides the bacteria with carbon because it contains organic compound for example coumarin. For some bacteria, coumarin can be a carbon source.[14]

Determining the bacteria for cultivation based on the growth kinetics for each isolate. According to the growth assesment it is known that BAP_5 isolate is the isolate that able to grow with the lowest generation time (g) which is 2.70 hour and the highest specific growth (μ) which is 0.26/ hour.

Cultivation of BAP_5 bacteria isolate in the medium added with purwoceng herbal infusion took place for 96 hour incubation. The isolate grew well in that minimum medium was indicated indicated by its growth pattern that did not show long lag phase.

Qualitative and quantitavie analysis of chemical compound in the culture medium by using TLC was for detecting the coumarin content in the medium at the beginning and end of incubation.

According to the *Rf* of coumarin standard, the compound spot of sample showing *Rf* 0.27 is the compound that has the same polarity with polarity of coumarin so it is presumed as coumarin. The compound spot with *Rf* 0.27 was shown by chloroform extract of inoculated and uninoculated medium for 0 hour incubation. It was indicated that coumarin or coumarin like compound is present in the medium and originated from purwoceng herba infusion that was added into the medium. Compound showing *Rf* 0.27 was still detected in the inoculated and uninoculated medium after 96 hour incubation.

Quantitative analysis of TLC profile showed that the decrease of coumarin within the uninoculated medium was higher than the decrease of coumarin in the inoculated medium. This result indicated that activity of BAP_5 bacteria isolate in the culture medium is able to keep the coumarin amount. The bacteria ability in keeping the coumarin content of the medium presumably due to the ability of bacteria in producing coumarin or using another organic compound within the medium as carbon source.

The role of endophytic bacteria in purwoceng plant organs probably by performing induction of coumarin production after colonization into roots and leaves. Another possibility of their role is providing aromatic amino acid (coumarin precursor) that can be produced by bacteria via shikimic acid pathway.[15] Therefore, it is recommended to do reinoculation of endophytic bacteria into sterile purwoceng plant to reveal the influence of endophytic bacteria to purwoceng especially in coumarin production.

In conclusion, endophytic bacteria were successfully isolated from purwoceng and the bacteria culture prevented the coumarin loss from the medium.

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