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บทความวิจัย

# การคัดเลือกและจัดจำแนกเชื้อแบคทีเรียที่สามารถผลิตโพลีไฮดรอกซีอัลคาโนเอตจากน้ำทิ้งโรงงานอุตสาหกรรมอาหารด้วยเทคนิคการย้อมด้วยสารเรืองแสง

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**บทคัดย่อ**

การคัดเลือกเชื้อแบคทีเรียที่สามารถผลิตสารโพลีไฮดรอกซีอัลคาโนเอต (Polyhydroxyalkanote; PHA) จากน้ำทิ้งโรงงานอุตสาหกรรมโดยใช้เทคนิคการย้อมด้วยสารเรืองแสง Nile blue A สามารถแยกเชื้อแบคทีเรียได้ทั้งสิ้น 16 ไอโซเลต จากการจัดจำแนกสายพันธุ์แบคทีเรียด้วยการศึกษาข้อมูลลำดับนิวคลีโอไทด์บริเวณ 16S rDNA ร่วมกับการศึกษาความสัมพันธ์ทางลำดับวิวัฒนาการ พบว่าเชื้อแบคทีเรียที่แยกได้สามารถจัดจำแนกได้เป็น 5 สกุล ประกอบด้วย *Bacillus*, *Enterobacter*, *Klebsiella*, *Asaia*, และ *Herbaspirillum* นอกจากนี้ผลการจัดจำแนกสายพันธุ์แบคทีเรียไอโซเลต OST-B2 และ OST-RA9 มีความใกล้เคียงกับสายพันธุ์ *Asaia bogorensis* และ *Herbaspirillum huttiense* ซึ่งยังไม่พบรายงานการผลิต PHA จากแบคทีเรียทั้งสองสายพันธุ์ จึงสามารถสรุปได้ว่า OST-B2 และ OST-RA9 เป็นแบคทีเรียสายพันธุ์ใหม่ที่พบการสะสม PHA ภายในเซลล์ นอกจากนี้ยังได้มีการศึกษาความสามารถในการสะสม PHA ภายในเซลล์แบคทีเรียที่คัดเลือกได้ทั้ง 16 ไอโซเลต ภายใต้สภาวะการเพาะเลี้ยงที่ 24, 48, และ 72 ชั่วโมง โดยวิเคราะห์การวัดค่าการดูดกลืนแสงฟลูออเรสเซนจากการติดสีย้อม Nile red ของ PHA แกรนูลภายในเซลล์ พบว่าแบคทีเรียไอโซเลต OST-RA9 สามารถสะสม PHA มากที่สุดโดยเฉพาะที่ 72 ชั่วโมงของการเพาะเลี้ยง ในขณะที่ไอโซเลตอื่นๆ มีการสะสมสูงสุดในช่วง 48 ชั่วโมง และจากผลการจัดจำแนกสายพันธุ์แบคทีเรียร่วมกับการศึกษาความสามารถในการสะสม PHA ภายในเซลล์ จึงทำให้ไอโซเลต OST-RA9 เป็นสายพันธุ์ที่น่าสนใจในการศึกษารูปแบบการสร้างและสะสม PHA ภายในเซลล์โดยการศึกษาภายใต้กล้องจุลทรรศน์อิเล็กตรอนชนิดส่องผ่านพบว่าเชื้อแบคทีเรียสามารถสะสม PHA ในลักษณะแกรนูลภายในเซลล์ ซึ่งผลจากการศึกษาในครั้งนี้ทำให้ได้ทราบถึงเชื้อแบคทีเรียชนิดใหม่ๆ ที่สามารถผลิต PHA ได้ และสามารถนำไปสู่การศึกษาแหล่งวัตถุดิบราคาถูกรวมทั้งการพัฒนาสายพันธุ์แบคทีเรียในการผลิต PHA ให้มีปริมาณสูงต่อไป

**คำสำคัญ**: โพลีไฮดรอกซีอัลคาโนเอต, แบคทีเรียผลิต PHA, เทคนิคการย้อมด้วยสารเรืองแสง, น้ำทิ้งโรงงานอุตสาหกรรม, สีย้อม Nile Red, สีย้อม Nile Blue A

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Research Article

# Isolation and Identification of Polyhydroxyalkanoate (PHA) Producing Bacteria from Food Industrial Wastewater by Using Fluorometric Screening

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**Abstract**

The sixteen polyhydroxyalkanoate (PHA) producing bacteria were isolated from industrial wastewater using Nile blue A fluorescence staining technique. The derived PHA-producing strains were identified through the 16S rDNA sequencing analysis and even phylogenetic evaluation. The results revealed that they were belonging to the five bacterial genera including *Bacillus*, *Enterobacter*, *Klebsiella*, *Asaia*, and *Herbaspirillum*. Moreover, the genetically identification profile of the two isolates OST-B2 and OST-RA9 were subsequently related to the strain *Asaia bogorensis* and *Herbaspirillum huttiense* which has not been proposed as the non-PHA accumulation strains before; hence, they might be classified as the novel PHA producing isolates. Additionally, the intracellular PHA concentration was determined after 24, 48, and 72 h cultivation through the spectrofluorometric technique. As follow, the PHA accumulation profile of OST-RA9 was slightly increased according to the extended cultivation time and maximize production at 72 h cultivation while another bacterial isolates gave the highest PHA production at reach 48 h. Thus, the isolated OST-RA9 could be considered as the attractive PHA producing strain through the highest PHA existence along with its identification appearance. Moreover, the distribution of intracellular PHA granules was subsequently explored in the isolate OST-RA9 according to the electron micrograph observation. Hence, the result would be exhibited a considerable activation of PHA production in different sources through the providing of the most appropriate and inexpensive precursor as well as strain improvement to further maximize the PHA yield.

**Keywords**: Polyhydroxyalkanoates, PHA-producing Bacteria, Fluorescence Staining Technique, Industrial Wastewater, Nile Red Dye, Nile Blue A Dye

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## 1. Introduction

Polyhydroxyalkanoates (PHAs) are biopolyesters with diverse structural granules completely synthesized by numerous bacteria due to the activity of unique enzyme, PHA synthases [1]–[3]. They are served as the intracellular compounds providing the survival energy in either starvation or environmental changing [4]. PHA granules are usually embedded by 5–8 units within the almost entire cell in order to the highest PHA level obtained [5]. Besides the particular properties in biodegradable, PHAs have been generated from the renewable substrates and considered natural material such as glycerol, the by-product of biodiesel production [6]–[8]. PHAs could be generally categorized into three groups depending on the length of their monomeric. Short-chain-length PHA (scl-PHA) is a polymer consisted of the monomeric repeat units up to 4–5 carbons. PHAs with 6–14 carbon units are grouped as the medium-chain-length PHA (mcl-PHA), while long-chain-length PHA polymers are composed of the monomer units up to 14 carbons [6], [9]. The wide varieties of the PHA monomers affected to the material properties that depends on the polymer composition, which has afforded useful in such varied industrial applications such as shampoo bottles and heat valves [10].

PHAs are normally found in several bacterial strains, for example *Alcaligenes*, *Pseudomonas*, *Bacillus*, and some species of photosynthetic bacteria, *Rhodobacter sphaeroides* under the depletion of the nutritional conditions [11]–[16]. In addition, PHA is an abundant granule occurring in the bacterium *Ralstonia eutropha* respond to the unbalanced growth conditions. Presumably, it could be considered as the model organism for PHA biosynthesis [17], [18]. Alternatively, the metabolic engineering could be manipulated to enhance the PHA production in such a potential bacterial strain like *Escherichia coli* [19], [20] and also the efficient industrial strain *R. eutropha* (formerly *Cupriavidus necator*) [21].

However, the PHAs have still received the intensive attention due to their completely biosynthetic and biodegradable without toxic waste production [22]. Hence, the isolation of sustainable PHA producing bacteria should be proceed for the further efficiency PHA production.

## 2. Materials and Methods

### 2.1 Isolation of PHA producing bacteria from industrial wastewater

Nine milliliter of Mineral salt medium [MSM; containing (in g/l): agar 15, $Na_2HPO_4 \cdot 7H_2O$ 6.7, NaCl 10, $KH_2PO_4$ 1.5, NH4Cl 0.1, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2$ 0.01, ferrous ammonium citrate 0.06, and 1 ml of trace elements] was inoculated with 1 ml of wastewater from the food product Industries located in Phra Nakhon Si Ayutthaya province, Thailand, and subsequently incubated at 30°C with short time orbital shaking. A serial dilution was performed in fresh liquid MSM medium to obtain the appropriate bacterial colonies. Shortly, each of 0.1 ml aliquot culture was plated onto solid MSM medium supplemented with 5 g/l of glucose [23]. The petri dishes were incubated at 30°C for 2–4 days. Colonies that develop on the solid medium were randomly picked depending on their differ appearances such as color, form, as well as edge. The selected colonies were then screened for PHA accumulation within the cell using Nile blue A staining technique [24]. The staining was examined using Olympus BX 51 fluorescence microscope equipped with the appropriate

microscope filter. The isolated stained with Nile blue A would be fluoresced bright due to their intracellular PHA granules visible within a cell.

**2.2 Identification of PHA producing isolate based on 16S rDNA gene sequence**

Total genomic DNA was extracted from the PHA producing bacterial strain using Genomic DNA extraction kit (Geneaid, Taiwan). The 16S rDNA sequence was then synthesized using the genomic DNA as a template with the two universal primers, 27F: 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R: 5' TACGGYTACCTTGTTACGACTT 3' [25]. The PCR was carried out with Taq DNA polymerase (Invitrogen, USA) and the reaction was conducted using PCR thermal cycle (Biorad, USA) with the following program: 1 min at 94°C, 0.5 min at 50°C, and 2.5 min at 72°C for 30 cycles followed by extension for 10 min at 72°C. A total amount of PCR amplification products were submitted to Macrogen Inc., Korea for complete DNA sequencing. 16S rDNA sequences were compared with those available in the GenBank, EMBL, and DDBJ databases using the gapped BLASTN program through the National Center for Biotechnology Information server (NCBI). Identification criteria was defined at the species and genus level in order to the similarity at $\geq$ 99 and $\geq$ 97 percent, respectively [26]. A phylogenetic tree was constructed by the dnadist algorithm in the PHYLIP package (version 3.695) and represented by a TREEVIEW (version 1.6.6) software.

**2.3 Spectrofluorometric monitoring of PHA accumulation**

PHA was monitored spectrofluorometrically with Nile red as a fluorochrome following a modification of the procedure of Berlanga and coworkers [23]. The isolated PHA producing bacteria were grown in a 50 ml flask containing nitrogen-limited MSM, glucose (5 g/l), and 0.5 µg Nile red dye (Sigma-Aldrich, USA), dissolved in dimethylsulfoxide. Liquid cultures were incubated at 30°C, 150 rpm for 24, 48 and 72 h. After that, a 1 ml sample was collected and then centrifuged at 15,000 rpm at room temperature. Pellets were washed twice with 1 ml of PBS (pH 7.0) followed by resuspended in 1 ml of 0.1 M glycine-HCl (pH 3.0). To allow high intensity relevant, the cell solution was incubated at room temperature in the dark for at least 2 h. The relative amount of PHA within the cells those indicated by the intensity of Nile-red orange fluorescence, was measured using LS 55 Fluorescence Spectrometer (PerkinElmer, USA). The fluorescence excitation and emission wavelengths of the stained cells in 0.1 M glycine-HCl (pH 3.0) were 543 nm and 598 nm, respectively. Slits of excitation and emission were set to 10 nm at 900 V [23].

**2.4 Transmission electron microscopy**

The highest PHA accumulation strain was selected for study the intracellular PHA granule using Transmission Electron Microscope (TEM). For excess PHA accumulation induction, the selected strain was cultured in a liquid MSM medium supplemented with 1% glucose and then cultured at 30°C, 150 rpm for 24 h. The cell was pelleted and washed with 1 ml of PBS (pH 7.0), followed by fixed with 2% glutaraldehyde. Accordingly, the fixed cell was then stained with osmium tetroxide and uranyl acetate. In order to complete TEM accomplishment, the sample was consequently submitted to the Scientific Equipment Center, Prince of Songkla University (SEC-PSU), Thailand.

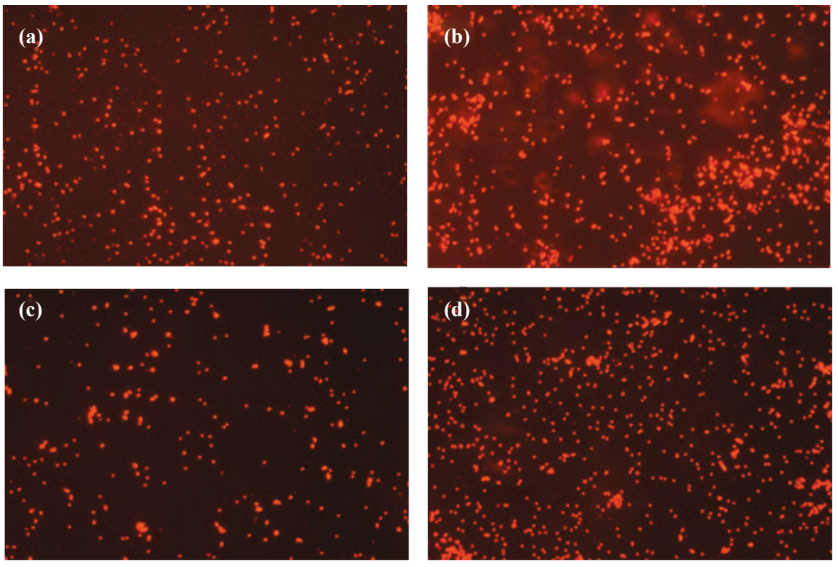


**Figure 1** Fluorescence microscopy of intracellular PHA granules of the isolates OST-ALF1-2 (a); OST-ALF2-1 (b); OST-AT2 (c); OST-AT4 (d), respectively. The PHA granules within the cells were exhibited the orange fluorescence brightness.

## 3 Results and Discussions

### 3.1 Isolation of PHA producing bacteria from industrial wastewater

The diverse isolated bacteria were subsequently screened for PHA production using Nile blue A staining technique. Among strains investigation, the sixteen isolates were identified as the PHA producing strains based on their strongly appeared bright fluorescent. The example of the stained cell showed strongly fluorescent was depicted in Figure 1, namely isolates OST-ALF1-2, OST-ALF2-1, OST-AT2, and OST-AT4, respectively. Thus, the fluorescent behavior detected strains could be considered as the PHA accumulation bacteria, because of the fluorescent dye binding to polymer granules within the cell [24], [27]. On the other hand, no fluorescent was observed with non-PHA producing isolates after completed with Nile blue A staining determination. The whole fluorescent bright isolates were then further analyzed for intracellular PHA accumulation.

### 3.2 Identification of PHA producing isolate based on 16S rDNA gene sequence

The total PHA producing bacterial strains were submitted to 16S rDNA sequencing analyzed. The data bank searched and alignment suggested that the overall bacterial strains shared the high similarity to the five bacterial genera including *Bacillus*, *Enterobacter*, *Klebsiella*, *Asaia*, and *Herbaspirillum*. Most of isolated bacteria were fell into a group of *Baciilus* involving *Bacillus* sp., *B. megaterium*, as well as *B. methylotrophicus*. Some of the isolated strains have been reported as the PHA producing bacteria, for example, *Enterobacter* [28] and *Klebsiella* [29]. Surprisingly, the isolate OST-B2 and OST-RA9 would be recognized as the novel PHA accumulating strains

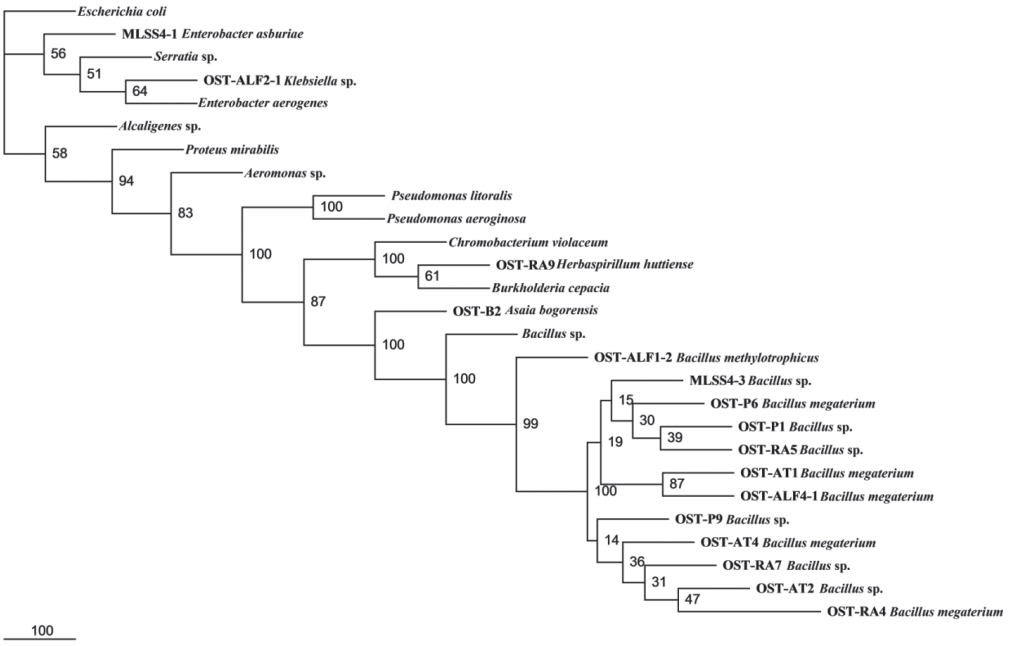


**Figure 2** Phylogenetic relationship of 16S rDNA gene sequences showing the inferred evolutionary relationships among the isolated PHA producing bacteria and the other genotypic bacteria. The tree was constructed based on the dnadist algorithm in the PHYLIP package. Bootstrap values are indicated as percentages at the nodes of branches.

due to their evidences from the highly homologous to the non-PHA producing strain *Asaia bogorensis*, and *Herbaspirillum huttiense*, respectively. Thus, the two isolated, OST-B2 and OST-RA9, might be continuously promote regarding to the high level intracellular PHA accumulation toward strain improvement or cultivation condition verification. In addition, the phylogenetic relationship of those PHA producing strain was constructed based on the alignment of the amplified 16S rDNA sequences among the sixteen isolated bacteria against to the published PHA producing bacteria. The genotypic evidence was illustrated in the Figure 2, the majority of isolated PHA producing bacteria were grouped by the cluster of *Bacillus*, while the other were divided along with the similar edge.

### 3.3 Spectrofluorometric monitoring of PHA accumulation

The PHA accumulation within the sixteen bacterial isolates was monitored over a cultivation time of 24, 48, and 72 h using the spectrofluorometric technique. The performing Nile red intensity was related to the amount intracellular PHA profile [23]. The PHA production was performed through the nitrogen-limited MSM medium supplemented with glucose (5 g/l), and 0.5 μg Nile red. The result suggested that the almost PHA-producing strains were completely accumulated the polymer at approximately 48 h especially the isolates OST-ALF2-1, OST-AT1, OST-AT2, and OST-RA5, while the isolates OST-P1, OST-P6, OST-P9 and OST-RA4 were strongly

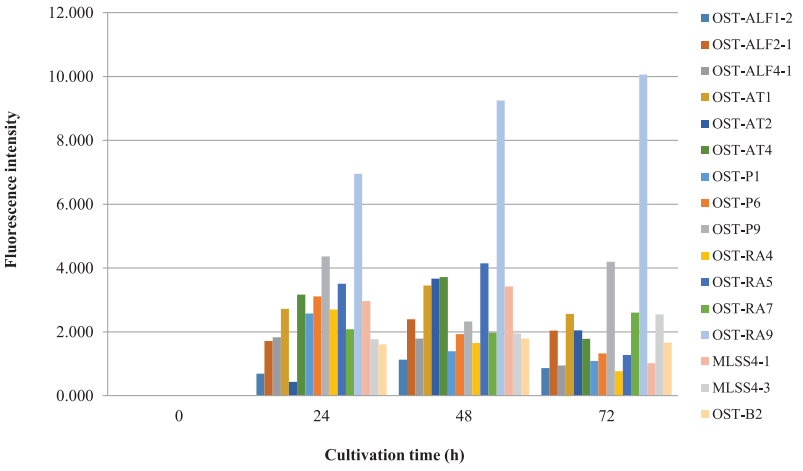


**Figure 3** The relative fluorescence intensity associated with intracellular PHA granule accumulation of the expected PHA-producing isolates. Experiments were carried out with three times cultivation composed of 24, 48 and 72 h, respectively.

exhibited the PHA production at 24 h cultivation. Interestingly, the highest PHA accumulation level of each cultivation time was existed in the isolate OST-RA9 particularly at 72 h incubation (Figure 3). Besides to the success in the intracellular PHA granules formation, the bacterial isolate OST-RA9 could be considered as the novel source for PHA production due to it recently exposed the empirical evidence against to the first report of PHA accumulation. So, it should be support to the genetic manipulation as well as the optimum culturing condition for extending the intracellular PHA level.

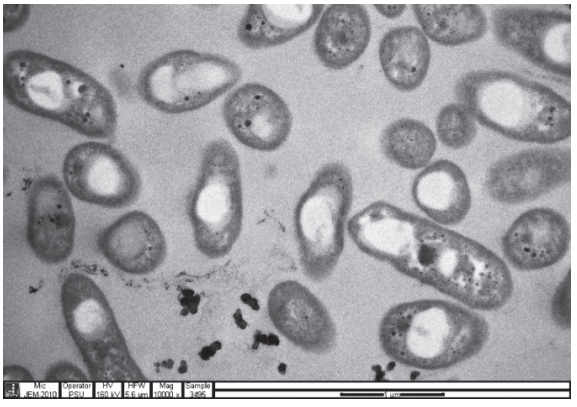

**Figure 4** Transmission electron micrograph of intracellular PHA granules in the bacterial strain OST-RA9. The indicating bar indicated 1.0 µm length.

### 3.4 Transmission electron microscopy

The distribution of PHA granules of the selected isolate, OST-RA9, was proposed within the bacterial cell as illustrated in the Figure 4. The familiar PHA accumulation behavior was significantly described in other PHA producing bacterial strains, for example, the present of electron transparent inclusions of *P. aeroginosa* due to the detection of lipid composition involved in the granule surface [30] as well as the demonstrated intracellular PHA inclusions over the electron microscope observation either in wild type or recombinant *R. eutropha* during stationary growth phase culturing in the modified MSM medium [31].

## 4. Conclusions

The PHA producing bacteria were totally derived from the food industrial wastewater along Phra Nakhon Si Ayutthaya Province, Thailand. The difference colonies as well as dissimilarity cell morphologies were selected to PHA accumulation isolation. The intracellular PHA accumulation was developed through the Nile blue A staining technique, yielding the sixteen isolates due to their orange bright under the fluorescence microscope observation. The overall sixteen bacterial isolates were identified through the 16s rDNA sequencing analysis using PCR amplification with the universal primer (27F and 1492R). The result proposed that, all of those sixteen bacterial isolates were grouped by the five genera including *Bacillus*, *Klebsiella*, *Asaia*, *Enterobacter*, and *Herbaspirillum*. In particular, the two identified isolates, OST-RA9 and OST-B2, were absolutely exhibited highly homologous to the bacteria *Herbaspirillum huttiense* and *Asia bogorensis*, respectively, implying that it might be the first report response to their PHA accumulation within the cells. Meanwhile, the intracellular PHA constituent was exposed using the fluorescence spectroscopy. It was summarized that an almost bacterial strains capable to generate the intracellular PHA within 48 h, while the PHA concentration was dramatically declined after 72 h cultivation. Additionally, the strain OST-RA9 was possessed the highest PHA accumulation together with its continually produced reach to 72 h cultivation time. Consequently, the transmission electron micrograph was then carried out to explain the distribution of biopolymer granules, the existence of internal PHA granules was ordinarily observed in the isolate OST-RA9. As a result, the derived PHA producing bacteria could be applied to enhance an alternative source for further industrial development, although the culture condition and also substrate variations should be optimized to yield the sustainable commercially PHA production.

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