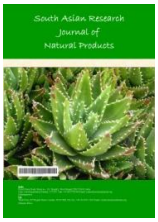

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Production of Ascorbic Acid by *Acetobacter* Species Isolated from Soil in Keffi, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author EJB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MDM, IKE and SOO managed the analyses of the study. Authors JEO and IHN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This investigation aimed at Production of Ascorbic acid by *Acetobacter* spp Isolated from soil in Keffi, was carried out using standard microbiological methods and identified standard techniques. The ability of the various isolates to produce Ascorbic acid were determined using starch solution Test and iodine crystal potassium iodide method while the quantitative determination of Ascorbic acid produced by each isolate was carried out and estimated using Gas Chromatography and Mass Spectrometry (GC & MS). The results obtained showed that the different species of *Acetobacter* isolated from the study area included *Acetobacter orientalis* and *Acetobacter orleanensis.* The investigation further revealed that *Acetobacter orientalis* produced the highest Ascorbic acid at temperature of 28^{o}C with 12.34 mg/l and least at 39^{o}C with 5.12 mg/l, while *Acetobacter orleanensis* also produced highest at 28^{o}C with 8.04 mg/l and least at 39^{o}C with 2.75 mg/l. *Acetobacter orientalis* yielded highest at 20% substrate concentration with 19.51 mg/l and least at 5% substrate concentration 4.19 mg/l, while *Acetobacter orleanensis* yield highest at substrate concentration of 25% with 10.98 mg/l and least at 5% substrate concentration 1.75 mg/l. *Acetobacter orientalis* produced highest ascorbic acid after 96 hours with 16.22 mg/l and lowest after 144 hours with 2.5 mg/l. *Acetobacter orleanensis* also produced highest Ascorbic acid after

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96hours with 10.11 mg/l and least after 144 hours with 1.02 mg/l. Highest ascorbic acid was produced at pH5.0 by *Acetobacter orientalis* with 16.68 mg/l and the least was at pH 4.0 with 3.21 mg/l while *Acetobacter orleanensis* produced highest at pH 5.5 with 9.12mg/l, the lowest at pH4.0 with 3.12 mg/l. This study shows that *Acetobacter* species isolated from the soil in Keffi metropolis has the ability to produce Ascorbic acid.

Keywords: Acetobacter; *gas chromatography and mass spectrometry; produced; ascorbic acid.*

1. INTRODUCTION

Ascorbic acid (L-AA) also known as vitamin C is a water-soluble vitamin that is indispensable for several physiological functions. It was first isolated from adrenal glands and subsequently characterized from plant tissues [1].

The discovery of L-AA is related with the disease of scurvy [2]. Scurvy was a common disease in the world's navies and sailors until the beginning of the nineteenth century, with serious symptoms such as bleeding of mucous membranes, anaemia and eventually death [3]. Vitamin C or L-ascorbic acid is an important metabolite for humans, non-human primates, and a few other mammals. In higher organisms, it is indispensable for different physiological functions and thus becomes an essential nutrient for animals (like humans) since they cannot synthesize this vitamin [4,5].

The Reichstein process was the industrial method used in producing Vitamin C. However, a modern two-step fermentation process is now employed in China, which produces 95% of the world supply of ascorbic acid [6]. In 2016, the Chinese government took regulatory actions that inflated vitamin C prices by up to 300%. The price of vitamin C rose three-fold to US $12 per kg as a result of the pressure on Chinese industry to discontinue burning coal which is an integral aspect of vitamin C manufacturing. The vast majority of L-Ascorbic acid is conventionally synthesized by a variety of known methods, which are generally variations of the Reichstein process [6]. However, these methods are mostly relatively complex chemical processes that involve the manufacture of ascorbic acid under conditions that require high temperature and pressures that are highly energy consuming as well as involving the use of organic solvents or strong acids or bases that constitute environmentally hazardous chemicals [7]. Giving rise to a manufacturing process burden with energy cost, economic and ecological problems. Moreover, the increasing demand and growing worldwide competition are the driving force for the development of alternative L-AA production methods [8].

Recent findings show that microbial production of L-AA simplifies the present L-AA production methods using different bacterial strains and this has been clearly established [9]. Therefore identifying indigenous bacteria capable of producing L-AA as well as its potential microbial input in the commercial synthesis of L-AA would be desirable. In this respect, a number of indigenous species from the soil will be investigated including members of the genera *Acetobacter*.

This project is targeted towards the exploitation of biological systems for the production of vitamin C. Thus it seeks to obtain L-Ascorbic acid producing bacteria in the local soil in Keffi city in Nasarawa State of Nigeria.

2. MATERIALS AND METHODS

2.1 Methods

2.1.1 Study area

This study was carried out in Keffi Local Government Area (LGA), Nasarawa State, Nigeria. Keffi is approximately 68km away from the Federal Capital Territory (FCT), Abuja and 128km away from Lafia, the Capital town of Nasarawa State. Keffi is located at longitude 8^{O}5'E along the Greenwich Meridian and at the equator and situated on longitude 850m above sea level [10].

2.1.2 Sample collection

Twenty soil samples were randomly collected from cassava farm/ cassava dump site and sugarcane farm/ sugarcane dump site at Angwan Lambu in Keffi Metropolis, using clean plastic containers and transported to the Microbiology Laboratory, Nasarawa State University, Keffi for analysis. Using a method described by [11].

2.1.3 Isolation of *Acetobacter* spp

The *Acetobacter* spp was isolated from soil in Keffi by the method earlier described by Amin et al. [12]. Briefly using this method, 1.0g of soil was suspended in 9.0ml of sterile distilled water

or sterile Ringer's solution and 10-fold dilutions was made and 1 ml of the aliquot was diluted in 9 ml of enrichment broth containing (0.5% glucose, 2% glycerol, 1% yeast extract, 1% peptone, 1.5% potato extract, 4% ethanol and cycloheximide which is an antifungal agent) and was incubated at 37^{o}C for 24hrs after which the 24hrs inoculum was streaked on GEY agar (Glucose, ethanol ,yeast extract agar), composed of 2% D-glucose; 0.8% yeast extract; 0.5% ethanol; 0.5% peptone; 0.3% CaCO3 and 1.5% agar supplemented with 10 mg/ml of cycloheximide (made up of 50% ethanol and 20ml/l of penicillin prepared from a 0.25% stock solution which inhibits the growth of yeasts and lactic acid bacteria, respectively) and incubated under aerobic conditions at 30°C for 1 week and further subcultured into Nutrient Agar (NA) plates and incubate 30^{o}C for 24 h . Isolates from GEY agar plates are maintained on agar slants of medium composed of 0.1% D-glucose, 1.5% glycerol, 0.5% peptone, 0.5% yeast extract, 0.2% malt extract, 0.7% CaCO3 and 1.5% agar as described by [13].

2.1.4 Identification of *Acetobacter* spp

2.1.4.1 Gram staining

The Gram staining of the presumptive organism was carried out as earlier described by [14]. Briefly, a smear of three (3) pure colonies of the organism was made on a drop of normal saline on a clean grease free slide and allowed to air-dry. The slide was passed twice through the flame to heat fix, then flooded with crystal violet solution for 30 sec and rinsed under slow running tap water. The washed slide was briefly decolorized with acetone, then immediately rinsed under slow running tap water and counter-stained with safranin solution for 60 sec. The slide was rinsed under slow running tap water, then allowed to air dry and the slide examined under x100 oil immersion objective if it appear pinkish or reddish color indicate gram positive.

2.2 Biochemical Tests

2.2.1 Indole test

The indole test was carried out as follows: three (3) pure colonies of presumptive *Acetobacter spp* was inoculated into 5 ml of peptone water in Bijou bottles and incubated at 37^{o}C for 24hrs. Two (2) drops of Kovac's reagent was added to 24hrs culture of the presumptive organisms. Formation of red ring at the top indicates indole positive reaction.

2.2.2 Methyl red test/ voges-proskauer test

Methyl red/Voges-Proskauer test on the presumptive organisms was carried out as follows: three (3) pure colonies of the suspected organism was inoculated into 10 ml of MR/VP medium in a Bijou bottle and incubated at 37^{o}C for 72hrs. The 72 hrs culture was divided into two portions. To the first portion, 2 drops of methyl red indicator was added and formation of red color was indicative of methyl red positive. To the second portion, ten drops of 10% potassium hydroxide was added, followed by 3 drops of beta-naphthol. Formation of pinkish red color was indicative of Voges-Proskauer positive.

2.2.3 Oxidase test

The oxidase test for suspected organism was carried out using slide method as described by [14]. Briefly, three (3) pure colonies of suspected isolates was smeared on a sterilized Watman paper soaked with 1% oxidase reagent and formation of purple color after 5 sec indicate oxidase positive.

2.2.4 Citrate test

The citrate test was carried out as follows: three (3) pure colonies of presumptive organisms was picked using sterile straight wire and stabbed on Simmons' citrate agar slant and incubated at 37^{o}C for 72hrs. Formation of blue color was considered indicative of citrate positive, and when it remains green then it is negative.

2.2.5 Catalase test

The catalase test for suspected organism was carried out using slide method as described by [14]. Briefly, three (3) pure colonies of suspected isolates were emulsified in a drop of 3% hydrogen peroxide on a glass slide and formation of bubbles was indicative of catalase positive.

2.3 16SrRNA Amplification

The 16SrRNA region of the rRNA genes of the isolates was amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microliters for 35 cycles. The PCR mix include: X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions was as follows: Initial denaturation, 95ºC for 5 minutes;

denaturation, 95ºC for 30 seconds; annealing, 52 ºC for 30 seconds; extension, 72 ºC for 30 sec for 35 cycles and final extension, 72 ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

2.3.1 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa.

2.4 Screening for Ascorbic Acid

Screening for ascorbic acid was carried out using the method described by [15], the presumptive *Acetobacter* spp was inoculated into a glucose broth using sterile wire loop and incubated at 37^{o}C for 48h. 2-3 drops of lugos iodine (iodine crystal potassium iodide) was then added. A positive result of the presence of ascorbic acid showed a change of color from blue-black to colorless after the fermentation media has neutralized the starch solution.

2.5 Ascorbic Acid Production

2.5.1 Inoculum preparation

The seed culture of the *Acetobacter* spp was prepared by modification of the method earlier described by [6]. Three (3) pure colonies of *Acetobacter* spp isolates from GEY agar was inoculated into 10 ml of Nutrient broth and incubated at 30^{o}C for 6hrs. The 6hrs Nutrient broth was further inoculated into 90 ml of fresh Nutrient broth in 250 ml Erlenmeyer flask and incubated at 30^{o}C for 18hrs in order to generate enough biomass for fermentation.

2.5.2 Fermentation

The production of ascorbic acid by *Acetobacter* spp isolates was carried using batch fermentation process by modification of method earlier described by [16]. Briefly, 100 ml of 18hrs inoculum was inoculated into 900 ml of Medium such as M1 (1.0% L-glucose, 5.0% baker's yeast, 0.05% glycerol, 0.25% $MgSO_4 .7H_20$, 1.75% corn steep liquor, 0.5% urea, 1.5% $CaCO_3$) containing different concentration of organic and nitrogen sources (5.0 g/L, 10.0 g/L and 20.0 g/L) and adjusted to different pH such as 4.0, 6.0, 8.0 and 10.0 respectively and incubated at different temperature such as 25^{o}C and 30^{o}C and 35^{o}C for 48 h, 72hrs and 96hrs in a shaker incubator at 240 rpm. The antifoam agent was added into the medium.

2.5.3 Quantification of ascorbic acid

The separation and quantification of ascorbic acid produced by *Acetobacter* species were carried out by Gas Chromatography and Mass Spectrometry (GC & MS) as described by [16] One microliter (1.0µL) of acidified sample was injected into "SHIMAZU GC-14, Gas Chromatograph" equipped with flame-ionization detector. The column used for the separation of solvent was PEG (2.1m x3.0mm). The temperature programming of the column oven was 60^{O}C/min 120^{O}C, Nitrogen gas (30 mL/minutes) was used as carrier gas. The temperatures of injector and detector were 150^{o}C and 200^{o}C respectively. The Peaks were recorded on "SHIMADZU C-R-4_A, Chromatograph", and were identified by comparison of the retention times with that of standard mixture. The experiment was carried out in duplicates and the means ± standard deviations of the yield of ascorbic acid were recorded.

3. RESULTS AND DISCUSSION

Table 1 shows occurrences of *Acetobacter* species from soil in Keffi, where the highest *Acetobacter* species was isolated from Cassava peel dump soil with 80%.

Table 2 shows the Cultural, morphological and biochemical characteristics of *Acetobacter* isolated from soil in Keffi.

Table 3 shows the isolates from different soil sample that were able to produce ascorbic acid during the screening.

Fig. 1 shows the effect of temperature on ascorbic acid production was observed at temperature of 28^{o}C where it yielded 12.34 mg/l and 8.04mg/l by *Acetobacter orientalis* and *Acetobacter orleanensis* respectively, this showed that temperature is an important parameter on ascorbic acid production and this is in agreement with the studies reported by [17] that best temperature for growth and metabolite production by *Acetobacter* spp is at 30^{o}C, also [18], reported similar thing saying that temperature play important role on ascorbic acid production using different microorganism.

Fig. 2 the effect of fermentation duration showed that ascorbic acid yielded highest at 96 hours of fermentation with Ascorbic acid yield of 16.22 mg/L and 10.11m g/L produced by *Acetobacter orientalis* and *Acetobacter orleanensis* respectively. Thus, 96hours was observed as the

optimum fermentation time for the ascorbic acid production which is similar to study reported by [19]. The yield of ascorbic acid reduced with increase in fermentation time for the two isolates, at 144 hours the yield of ascorbic acid by *Acetobacter orientalis* was 2.5mg/l and *Acetobacter orleanensis* was 1.02mg/L. This showed that ascorbic acid has been slowly degraded which means it also serve as source of carbon to the isolates or it can be converted to other organic acids.

The use of *Acetobacter* species in the production of ascorbic acid witnessed an increase in the yield of ascorbic acid when pH was at 5.0 (i.e, the lower the pH, the higher the quantity of ascorbic acid produced. In other words, the yield of ascorbic acid was favored by an increase in acidity). This study is consistent with the report published by [20,21], which says that the best growth was observed at pH range of 5-6 for organic acid production. The pH of the fermentation medium directly influences the growth of microorganisms and the biochemical processes they perform. It was observed that the yield of ascorbic acid obtained at pH5.0 was 16.68mg/l, at pH5.5 was 15.43mg/l by *Acetobacter orientalis* and at pH5.5 was 9.12mg/l, at pH6.0 was 8.89mg/l produced by *Acetobacter orleanensis*. However, ascorbic acid production reduced drastically to 3.12mg/L for both bacteria as the pH of the medium reduced to pH 4.

Substrate concentration has a significant effect on organic acid production. Most bacteria have high sugar uptake ability which helps in the acumination of the organic acid such as ascorbic acid in the fermentation medium [22]. In this study it was observed that the highest production of ascorbic acid was obtain at 20 and 25% Substrate concentration and the yield was 19.51 and 18.62 mg/l produced by *Acetobacter orientalis* while at 25 and 20% Substrate concentration, yield was 10.98 and 9.42mg/l by *Acetobacter orleanensis*, this showed that *Acetobacter* species are among the group of bacteria that are have the ability of converting sugar to other forms of organic acid. This is in agreement with the study reported by [23]. It was observed in this study that at all concentrations of substrates; the ascorbic acid yield varies from one concentration to another given highest yield at 20 and 25%. From this study it has also been established that fermentation parameter is of great important in organic acid production such as in ascorbic acid production by *Acetobacter* species isolated from soil in Keffi.

Table 1. Occurrences of bacteria species from soil in Keffi

Sample	No sample	No isolates	% isolates
Cassava farm soil	5	2	40.0%
Sugar cane farm soil	5	3	60.0%
Cassava peel dump soil	5	4	80.0%
Sugar cane peel dump soil	5	3	60.0%
Total	20	12	60.0%

Table 2. Cultural, morphological and biochemical characteristics of *Acetobacter* isolated from soil in Keffi

Cultural morphology	Gram stain	Biochemical characteristic				Sugar fermentation			Inference
		Cat	Ox	In	Nit	Fru	Mal	Glu	
Large, circular, regular and milky white on GYA	-	+	+	-	-	-	-	+	*Acetobacter* species

Key: cat= catalase; Ox= oxidase; In= indole; Nit= nitrate; fru= fructose; mal= maltose; Glu= glucose; += positive; -= negative

Table 3. Screening of ascorbic acid production by bacteria isolates

Isolates	Production of ascorbic acid
Scfp1	+
Scf2	-
Scf3	-
Csf 1	-
Csf 2	+
Scpa	+
Scf1	+
akcf2	-
akcf1	+
Csdp2	-
Csdp3	+

KEY: Scfp1 Sugarcane farm soil, Scf2 Sugarcane farm soil, Scf3 Sugarcane farm soil; Csf1 Cassava farm soil, Csf2 Cassava farm soil; Scpa Sugarcane peel dump soil; Scf1 Sugarcane farm soil; akcf2 Sugarcane peel dump soil, akcf1 Sugarcane peel dump soil; Csdp2 Cassava peel dump soil and Csdp3 Cassava peel dump soil

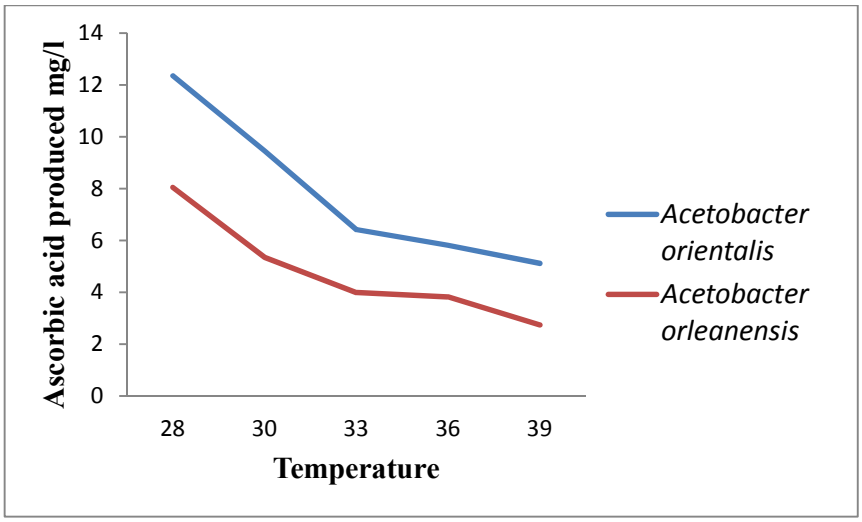

Fig. 1. Effect of Temperature on Ascorbic acid production

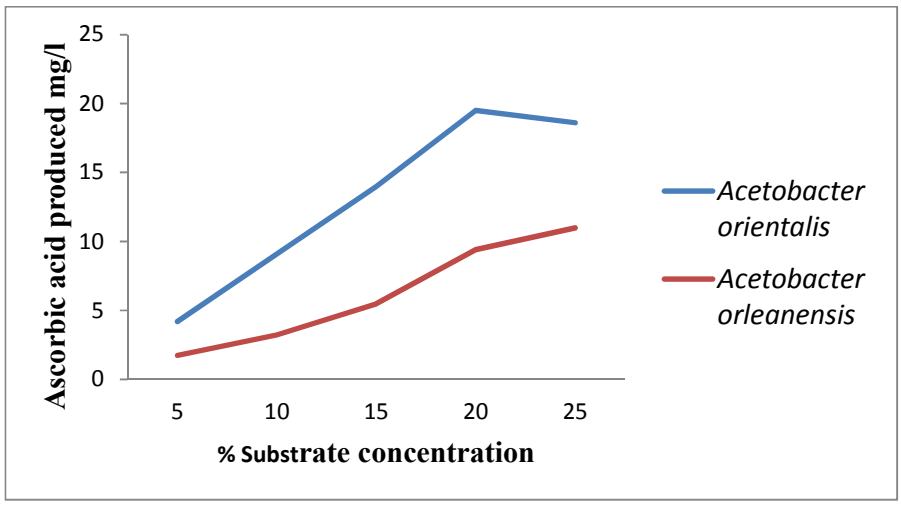

Fig. 2. Effect of substrate concentration on ascorbic acid production

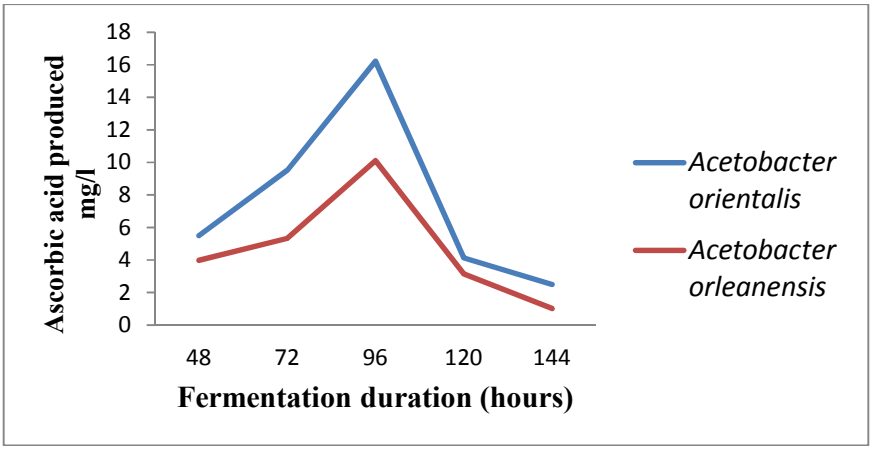

Fig. 3. Effect of fermentation duration on ascorbic acid production

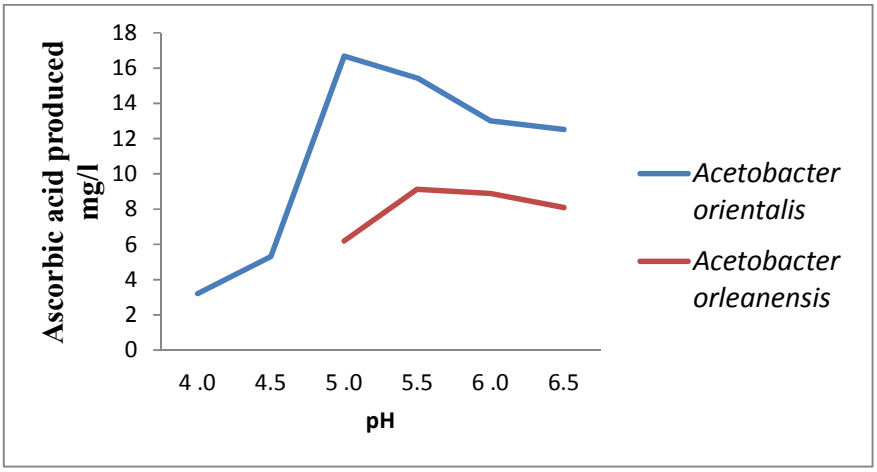

Fig. 4. Effect of pH on ascorbic acid production

4. CONCLUSION

There was high occurrence of *Acetobacter* species from dump site of both cassava peel and sugar cane peel in the study area. The *Acetobacter* speices isolated were Acetobacter *orientalis* and *Acetobacter orleanensis* all were able to produce ascorbic acid at different fermentation parameters which is important tool in the production of this organic acid by *Acetobacter* species isolated from soil in Keffi.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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