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Research article

Microbial production of ferulic acid and optimization of the medium parameters using fractional factorial design

Swati P Bhavsar*, Lokesh P Sharma

H.P.T. Arts and R.Y.K. Science College, Nasik, Maharashtra, India

ABSTRACT

Phenolics are widely distributed in plant kingdom and are therefore, an integral part of the diet, with significant amounts being reported in vegetables, fruits, and beverages. Various phenolic compounds have attracted the attention of food and medical scientists because of their fragrance, aroma, antioxidant, anti-inflammatory properties and ability to combat human diseases. Of these, Ferulic Acid (FA), a hydroxy cinnamic acid (related to trans-cinnamic acid), being natural, is of great demand in the food industry. As a component of lignin, FA is a precursor in the manufacture of other aromatic compounds. In our study, FA was produced using *Lactobacillus* spp. isolates and standard culture of Lactobacillus plantarum ATCC 8014. FA was extracted and partially characterized using Thin Layer Chromatography (TLC), Absorption maxima (λ_m) analysis and High Performance Liquid Chromatography (HPLC). Further, optimization of the fermentation medium was done using Factorial Fractional Design (FFD). Preliminary confirmation of the extracted FA was done using TLC, spectral analysis and purity assessed by HPLC. FA could be produced using Lactobacillus sp. and agro industrial waste viz., wheat bran, leading to a cost-effective protocol and product. Further, medium was optimized for the production of FA using FFD and it was observed that medium containing5.75% Wheat bran & 0.18% Tween 80 is optimum for the production of FA. The antimicrobial activity of FA was noteworthy against Aspergillus flavus and E. coli.

Keywords: Ferulic acid, *Lactobacillus* spp., Production, Optimization, Fractional Factorial Design (FFD)

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Correspondence: Dr. Swati Padmakar Bhavsar* ✉s wati.p.bhavsar@gmail.com

H.P.T. Arts and R.Y.K. Science College, Nasik -05, Maharashtra, India

INTRODUCTION

Commelinid plants (rice, wheat, oats, and pineapple), grasses, grains, vegetables, flowers, fruits, leaves, beans, seeds of coffee, artichoke, peanut and nuts commonly contain ferulic acid (FA)[1]. Around 0.5–2% extractable amount of FA is present in cell walls of cereal grains and a variety of food plants such as pineapple, bananas, spinach, and beetroot contains. FA is mostly in the trans-isomeric form, and esterified with the specific polysaccharides [2].

FA, can of scavenges &neutralizes free radicals. FA can also inhibit the enzymes required for catalyzing the production of free radicals. Thus, FA can be used as Antioxidant, Antidiabetic, and anti-ageing, anti-cancer, antimicrobial, anti-inflammatory and food preservative agent. FA can also be used as cardio vascular protective agent since it increases endogenous antioxidant defense, marker enzyme, inhibits platelet aggregation, and prevents thrombus formation. It can also be used as Precursor of vanillin since it can be converted into feruloylS CoA using ATP [3].

Apparently, there is need of the work related to extraction of bioactive phytochemicals which would prove an effective therapeutic and preservative agent. This can be achieved using inexpensive source such as wheat bran along with beneficial microorganisms. There are very scanty reports on production of FA using microorganism. In present work, we emphasize on using *Lactobacillus* spp. in the production and optimization of FA.

MATERIALS AND METHODS

Bacterial cultures growth and maintenance

Lactobacillus plantarum ATCC 8014 was obtained from NCIM, NCL, India (Grenobal Cedex, France). It was grown on de Man, Rogosa and Sharpe Medium, MRS (Hi Media, Mumbai) and incubated for 5 days at 37ºC in an anaerobic condition with CO_2 enriched atmosphere, using anaerobic gas pack (Hi Media). After incubation, the culture was stored at 4°C in refrigerator. Similarly *Lactobacillus* spp. isolated from home-made curd and *Drosophila melanogaster* (DM isolate from gut) and characterized using biochemical characteristics was maintained on MRS Medium [4].

Screening of isolates for Ferulic Acid Esterase Activity (Plate Assay Method)

One colony from the MRS-agar plate was inoculated into 5 ml of MRS broth and incubated at 37°C for 3 days. The fresh inoculum was further transferred to MRS broth containing 1.33 mM Ethyl Ferulate (EFA) and incubated under appropriate conditions. MRS-EFA agar plates were prepared by adding 0.3 ml sterile EFA into 10% dimethylformamide and further mixed it with 20 ml of MRS. Bacterial cultures were indigenously loaded on Whattman paper filter discs and eventually placed on the MRS-EFA agar, further incubated at 37°C in CO_2 enriched atmosphere, using anaerobic gas pack (Hi Media Mumbai)for 3 days. FA was detected and confirmed using chromatographic techniques (Thin layer chromatography and High performance liquid chromatography) [5].

Production of Ferulic Acid using *Lactobacillus* spp

The two *Lactobacillus* isolates and *Lactobacillus plantarum* ATCC 8014 were grown in MRS medium containing wheat bran. The pH of the medium was adjusted to 6.5 before autoclaving at 121°C for 15 minutes. The cultures were incubated for 5 days at 37°C in a CO_2 enriched atmosphere in an anaerobic condition and analysed for the quantity of ferulic acid. This experiment was set in triplicates [6].

Optimization of Ferulic acid production using Fractional Factorial Design (FFD)

Analysis of variance, statistical validity of the model and lack of fit of model for selected factorial model was determined using statistical software design expert (Version 11). A two-level FFD was used to determine the effect of wheat bran and tween-80 on the production of ferulic acid so as to standardize both the parameters. The cultures were grown in MRS medium containing wheat bran and tween 80and incubated for 5 days at 37°C in a CO_2 enriched atmosphere in an anaerobic condition. Twelve runs of experiments were designed, Wherein, wheat bran as X in high (6%) and low (3%) values and tween 80 as Y in high (0.5%) and low (0.1%) values were maintained. After appropriate incubation time, amount of ferulic acid produced was calculated as a response [7, 8].

Antimicrobial activity of Ferulic acid produced by *Lactobacillus* spp.

Twenty four hours grown culture of *Escherichia coli* (10^9 cfu/ml) was spread on Muller Hinton Agar plate. Ferulic acid dissolved in 50 % methanol was introduced in the well (5 mm) and incubated at 37°C for 24 hours whereas 50% methanol was kept as a control. After incubation, zone of inhibition was observed and measured [9-11].

Similarly, 72 hours grown culture of *Aspergillus flavus* (10^6 spore/ml) was spread on Sabouraud Dextrose Agar. Ferulic acid dissolved in methanol was introduced in the well (5 mm) and incubated at 37°C for 24-48 hours whereas 50% methanol was kept as a control. After incubation, zone of inhibition was observed and measured [12-14].

RESULTS

Ferulic Acid Esterase Activity using Plate assay

Ethyl Ferulate (EFA) clearing zones due to Ferulic Acid Esterase (FAE) activity, resulting in ferulic acid production were visible for standard culture of *Lactobacillus plantarum* ATCC 8014, *Lactobacillus* sp. isolated from *Drosophila melanogaster* and *Lactobacillus* sp. isolated from curd.

Production of Ferulic acid by *Lactobacillus* spp.

Three indigenous isolates of Lactobacillus spp. and the standard culture were inoculated in MRS medium and incubated for 5 days at 37° C, release of ferulic acid was estimated in triplicates and results represent the mean (Table No. 1)as follows:

Table 1: Ferulic acid released from de-starched wheat bran by *Lactobacillus* spp.

Lactobacillus isolates	Amount of Ferulic acid (mg/ml media)*
L. plantarum ATCC 8014	49±0.76
Lactobacillus sp. from Curd	30±0.57
Lactobacillus sp. from DM	42±0.19

* Values represents mean of amount of FA

Fractional Factorial Design Analysis (FFD)
Ferulic acid production using Wheat Bran and Tween 80

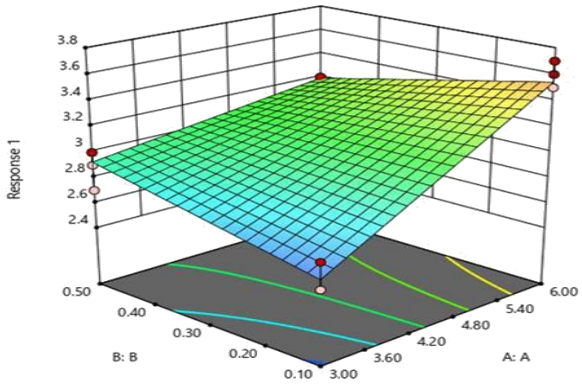
Analysis of variance, statistical validity of model and lack of fit model for selected factorial model was determined using statistical Software Design Expert (Version 11), INC, and USA. In this study, FFD design was used to find optimum condition for factors affecting FA released. Twelve experiments were performed (Table No. 2). Results obtained indicate the model F value 42.2 with error 0.01% at 0.0001% probability, implies that the larger F value would occur due to noise. Also, calculated F value is more than the tabulated F value at 1% level of significance. The null hypothesis is rejected which signifies that, wheat bran (3-6%) and Tween 80 (0.1-0.5%) significantly affects the FA production. The predicted R^2 of 0.8287 is in reasonable agreement with the adjusted R^2 of 0.8287 i.e. the difference is less than 0.2 adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio in current investigation is 14.462 which indicate an adequate signal. This model can be used to navigate the design space. The coefficient estimate represents the expected changes in response per unit change in factor value. When all the other remaining factors are held constant, the intercept in an orthogonal design is 3.06 with 0.038 error and is overall average response of all the 12 runs. The Variance Inflation Factor (VIF) is one, which is less than 10 indicating that factors are independent and not correlated. The 2 D contour and surface plot for optimization of FA production was based on regression equation. The graph No. 1 & 2 shows that optimum point for highest total FA production 3.7% (0.37gm/ml), when the optimum wheat bran concentration being 5.75% (0.575gm/ml) and Tween 80 is 0.18%

(V/V).

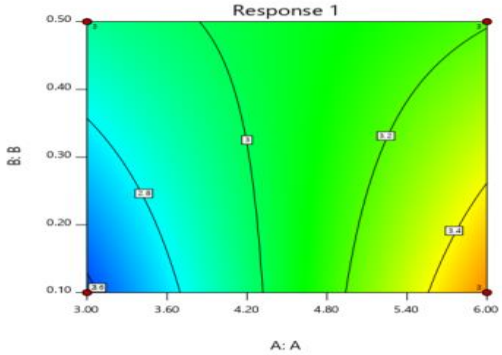
Table 2: Optimization of ferulic acid production by FFD

Run	Factor I Wheat bran (mg/l)	Factor II Tween80 (ml/10ml)	Response (%)
1	3	0.1	2.5
2	3	0.5	3
3	3	0.1	2.7
4	6	0.5	3.2
5	6	0.1	3.5
6	3	0.5	2.9
7	6	0.1	3
8	3	0.5	2.7
9	3	0.5	2.7
10	6	0.1	3.7
11	6	0.5	3.2
12	6	0.1	3.6

Graph 1: 3D Surface graph of FFD analysis



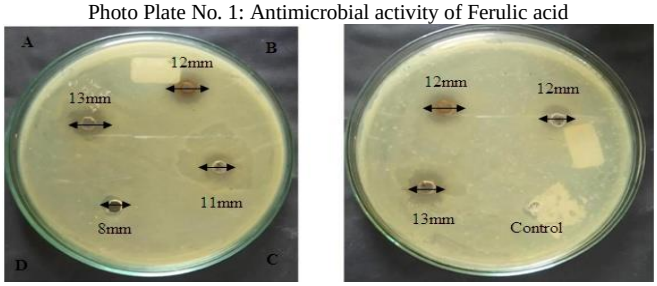
Graph 2: Counter graph of FFD analysis



Antibacterial activity of Ferulic Acid

Antimicrobial activity of extracted FA from different species of Lactobacillus was determined against the opportunistic pathogen, E. coli. The zone of diameter in between 7 to 13mm was observed (Photo Plate No. 1)

Photo Plate No. 1: Antimicrobial activity of Ferulic acid



(A) **(B)**

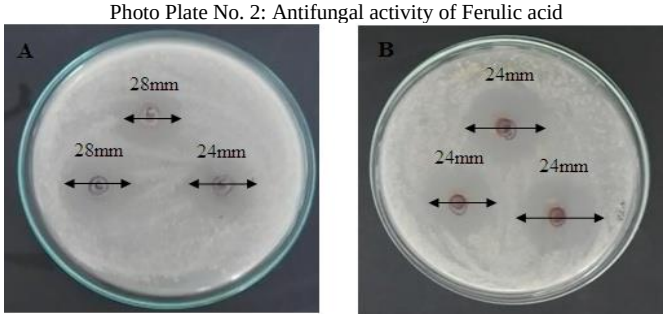
(A) Antibacterial activity of ferulic acid extracted from L. plantarum ATCC 8014

(B) Antibacterial activity of ferulic acid extracted from Lactobacillus isolate

Antifungal Activity of Extracted Ferulic Acid:

Antifungal activity of extracted FA from standard *Lactobacillus* sp and *Lactobacillus* isolate was observed against the *Aspergillus flavus* fungus. The zone of diameter about 24 to 28 mm was observed (Photo Plate No. 2)

Photo Plate No. 2: Antifungal activity of Ferulic acid



(A) (B)

(a) Antifungal activity of FA extracted from *L .plantarum* ATCC 8014

(b) Antifungal activity of FA extracted from *Lactobacillus isolate*

DISCUSSION

The most common microorganisms used in probiotic formations are the lactic acid bacteria, which are important component of the health gut, microbiota and have been regulated as safe by the American FDA, shown unique advantage in metabolic disease through the production of useful enzymes that exerts diseases, bio activities in the host.

Current investigation focuses on using specific bacterial strains that produce biologically active products. In recent studies**,** the products Cinnamyol esterase have shown remarkably high levels of antioxidant activity and other health benefits like stimulation of insulin secretion, prevention of oxidative stress, cholesterol lowering capabilities [15, 16].

FA exhibits antioxidant activity in response to free radicals via donating one hydrogen atom from its phenolic hydroxyl group, as a result it shows a strong anti- inflammatory activity in a carrageenan rat paw edema model and other similar systems. It is found that the antioxidant capacity of the phenolic acid was observed to be equivalent to lecithin upon comparison with ghee which was observed on inhibition of time dependent peroxide value.

In our study, curd sample was selected for isolation of Lactobacillus as it is commonly found in the fermented dairy products [17]. Curd being one of the fermented dairy products, serves as the rich as well as inexpensive source of *Lactobacillus* spp. The culture obtained from *Drosophila melanogaster* was revived and used for the production FA, it is because of the presence of esterase gene

been reported earlier in *Lactobacillus* isolated from *Drosophila melanogaster* gut. It may have happened that in the due course of co-evolution, the gene for esterase involved in producing FA must have been acquired by *Drosophila melanogaster* [18].

FA is one of the desired products of hydrolysis by Feruloyl Acid Esterase (FAE). Gut bacteria have been described as having FAE activity. So, for preliminary confirmation of production of FA, we focused on the strains for inherent FA production ability. Plate assays were performed and the principle behind it being the precipitation of FA by the activity of FAE, resulting in the hydrolysis of cinnamyol esters. As expected, FA clearing zones due to FAE activity were obtained and they were much more prominent for the *Lactobacillus* sp. isolated from *Drosophila melanogaster*. Similarly comparable zone of clearance measuring 10 mm was seen for L. fermentum NCIMB 3221. This primarily confirmed production of FA.

Wheat bran being a major milling byproduct is rich in arabinoxylans (AX). Enzymatic upgrading of bran is an attractive alternative to environmentally damaging chemical methods, currently used for lignocellulose saccharification. Wheat bran obtained from market contains 0.16% of total sugar (dry matter), polysaccharides (66%), Lignin (12%), protein (6%), Ash (4%), moisture (12%). Before using, it was de-starched for easy release of FA, which acts as an inducer. Tween 80 is a detergent which increases permeability in the cells, induces organisms to uptake the media components and in this way induces the organism to give desirable product (FA) [19].

The highest production of FA was observed to be 49 mg/ml using L. plantarum ATCC 8014. But, the HPLC analysis result revealed that FA extracted from Lactobacillus sp. isolated from Drosophila melanogaster is more pure than that of L. plantarum ATCC 8014 and the concentration was 0.282mg/ml, as compared to the reported value being 0.15221mg/ml L. fermentum ATCC 1196. According tovariance, statistical validity of the model was determined using the software design expert. In this study, FFD was used to find the optimum condition for factors affecting FA production. Twelve experiments were performed and results indicated that the model F value 42.2 with error 0.01% at 0.0001% probability implies that such a large F-value would occur due to noise. Also calculated F value is more than the tabulated value at 1% level of significance. The null hypothesis is rejected signifies FA production. The intercept in an orthogonal design is 3.06 with 3.08 errors showed that 5.75% wheat bran and tween 80 is 0.18% of the total medium. Once the media is optimized, physical parameter can then be optimized. In one such optimization the result showed that the order of contribution of physical factor towards FA is as follows; pH > type of co culture > volume of inoculum > agitation >fermentation time > temperature > water to substrate ratio [20].

CONCLUSION

The antimicrobial activity of FA was then studied against fungus and bacteria and from the experiment we came to the conclusion that FA has inhibitory on A. flavus and E. coli at concentration of 49µg/10µl being the highest one. The phytochemicals such as FA can be a promising agent in antimicrobial therapy. FA exhibit antioxidant activity in response to free radicals via donating one hydrogen atom from its hydroxyl group, as a result it shows strong anti-inflammatory activity in a carrageenan induce rat edema model and other similar systems. FA protects the membrane by successfully quenching of free radicals from attaching the membrane. It also exhibits the leakage of marker enzymes which can be studied in future.

The use of combinatorial and parallel synthetic chemistry techniques, high throughput screening and computational design will spread the discovery and effective product to treat resistant infection. Immediate practical application of FA can be proposed for antiseptic and disinfectant formulations due to that recognized cutaneous toxicity

On the basis of our work, we conclude that FA can be extracted using bacteria such as Lactobacillus sp. and an agro industrial waste containing wheat bran. This results in an inexpensive technology giving useful product. The steps can be modified or altered for simultaneously producing other beneficial products like ascorbic acid to be used as therapeutic agent.

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