



Submerged fermentation as a suitable solution to produce humic and fulvic acids from sugarcane bagasse

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Abstract. Humic and fulvic acids are valuable compounds that are produced from sugarcane bagasse using Solid-State Fermentation (SSF) and Submerged Fermentation (SmF). Initially, fourteen treatments were examined to determine the most prominent one with a proper C/N ratio. BFFU (Bagasse + Filter cake + Fungi booster + Urea) is regarded as the most effective mixture. The results obtained from SSF using BFFU treatment included 3.96 and 2.36% dry matters for Humic Acid (HA) and Fulvic Acid (FA), respectively. Optimal treatment and fermentation systems were implemented using two different SmF bioreactor volumes (60 and 1000 liter) to determine the optimal condition for higher yield. In contrast with the SSF, production yield in the SmF system resulted in a higher yield within a shorter processing time (36 days in SmF instead of 70 days in SSF). These obtained values were almost three times more than the SSF results in the range of 12.84 to 7.91% DM over a period of 36 days. However, the production rate was slightly higher when using a 1000-liter Continuous Stirred-Tank Reactor (CSTR) (14.25 and 8.39% DM over 35 days) than that in regular conditions. The results from Fourier Transform Infrared Spectroscopy (FTIR) pointed to some structural similarities between the SB-derived HA and FA in the present study and those of their commercial counterparts.

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1. Introduction

Routine use of chemical fertilizers causes several problems such as Organic Carbon (OC) reduction, acidification, and more stability problems that threaten both human health and environment, especially the soil structure and fertility [1,2]. As reported earlier,

the crop uptake of inorganic fertilizers is only 30–50%, and the residual components pollute groundwater by over-accumulation of chemical elements [3]. Therefore, it is necessary to substitute these chemicals with more reliable compounds, e.g., organic fertilizers, to find solutions to soil quality and fertility deficiencies [4]. Highly organic soil provides soil-dwelling microorganisms with an improved decomposing ability to consume carbon and nitrogen elements and produce compost abundant in humic substances such as Humic Acid (HA) and Fulvic Acid (FA) [5,6]. HA is commonly used to improve both soil fertility and plant growth rate owing to its ability to absorb soil particles, im-

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Table 1. Comparison of HA and FA production in this study with other findings.

Substrate	Production method	HA	FA	Process time (day)	Ref.
Pineapple leaf residue	Ready-made compost is used for analysis	20% DM	–	–	[50]
Activated sludge and green waste	SSF	3.97% DM	–	60	[53]
Wheat straw with low-grade rock phosphate	SSF	13.8% OM	2.68% OM	120	[54]
Empty fruit bunch	SSF	0.327% DM	–	49	
Rice straw	SSF	1.09% DM	–	47	
Rice husk	SSF	0.313% DM	–	49	
Sawdust	SSF	0.423% DM	–	42	[55,56]
Vermicompost from green wastes	SSF	1.4% DM	–	55	
Biogas residue, swine manure, chicken manure, and maize straw	SSF	1.95% DM	4.27% DM	37	[57]
Pruning waste, leaves, and grass clippings	SSF	2.84% DM	0.99% DM	145	[58]
Soil amended with SB	SSF	2.78% DM	–	30	[52]
Sago bagasse, chicken feed, molasses, and chicken manure slurry	SSF	10% DM	–	57	[59]
	SSF scale 220 L	3.96% DM 10.9% OM	2.36% DM 6.4% OM	70	
SB, filter cake, Fungi booster, and urea	SmF scale 60 L	12.84% DM 35.3% OM	7.91% DM 21.7% OM	36	Present study
	SmF scale 1000 L	14.25% DM	8.39% DM	35	

prove drainage, and provide spaces between particles that act as a reservoir for water and air, i.e., all the requirements for the plant growth [7–9]. Not only do organic fertilizers ensure better quality for agricultural products, but also they are cost-effective [10]. To the best of our knowledge, the number of published papers on the optimization of the HA and FA production using Submerged Fermentation (SmF) is rare, as shown in Table 1. Difficulty in lignocellulose breakdown is the main cause of this problem; hence, a great need for developing a cost-efficient technology is felt to overcome this problem [11]. In the sugar production process, different by-products are produced

called bagasse [12]. The produced Sugarcane Bagasse (SB) has a hard structure due to the presence of lignocellulos composition, which causes a major challenge in biodegradation and conversion into valuable products [13]. There are several factors that nominate SB as a valuable compound in industry including the high annual production rate, accessibility, and economic feasibility [14]. Therefore, production of HA and FA from SB can be a proper solution with the lowest risk to the environment and economy and also, be useful for agricultural fertility [15]. The main objective of this study is to develop different fermentation systems equipped with different treatments and a fungal

booster to calculate the quality and quantity of HA and FA production from SB. All prominent parameters are monitored during the process to ensure the accuracy of the process. In addition, in order to achieve the highest yield, the process is implemented in two pilot-scale volumes and the proper volume for a higher production rate is determined.

2. Materials and methods

2.1. Sampling

The SB and filter cake were prepared three times within a two-week interval in Salman Farsi Agro-Industry in Khuzestan Province, Iran. Once dried, their smaller particles with a diameter of 5 mm were formed by grinding and sieving. Poultry manure was also collocated from poultry farming. The compost was then collected from the Aradkooch recycling factory in Kahrizak, Tehran. In addition, the sewage sludge was obtained from a sludge and sewage treatment plant in Karaj, Iran.

2.2. Treatments: design and preparation

The current study employed a full-factor statistical design with 28 SmF and SSF (solid State Fermentation) treatments (14 SSF and 14 SmF treatments). The examined variables include (1) the amounts of Total Solid (TS) at two levels of 30% and 10% which are called SSF and SmF, respectively; (2) the microbial inoculation at four levels namely cow manure, compost, activated sewage sludge, and a fungal booster; and (3) the urea and poultry manure used as a nitrogen source for C/N regulation. Table 2 presents the treatments with their C/N ratios. Each treatment is performed in two repetitions. The ratio of C/N in both SSF and SmF

treatments is the same, thus making the comparison of their processes more possible. Given that the best C/N ratio for decomposition and fermentation processes ranges between 30–35 [16], the C/N ratio of 32.5 was adjusted for all treatments using the formula given below, which is based on the carbon and nitrogen content of different compounds [17].

$$\frac{C}{N} = \frac{[\%Ca \times a \times (1 - Ma)] + [\%Cb \times b \times (1 - Mb)] + \dots}{[\%Na \times a \times (1 - Ma)] + [\%Nb \times b \times (1 - Mb)] + \dots}, \quad (1)$$

where Ca is carbon content of compound a; Cb carbon content of compound b; Na nitrogen content of compound a; Nb nitrogen content of compound b; a weight of compound a; b weight of compound b; Ma moisture of compound a; and Mb moisture of compound b.

In this study, the “bagasse” and “bagasse + filter cake” were used as the control group without C/N ratio regulation. In addition, the treatments “bagasse + filter cake + nitrogen source (urea or poultry manure)” were used as the control group without the addition of microbial inoculation. All the designed treatments for both SSF and SmF are listed in Table 2. In all of the listed treatments, a mixture of SB and filter cake at a ratio of 3:1 was used. Here, the precise amount of SB and filter cake including 12 and 4 kg Dry Matter (DM) was put into 220 L barrels in the SSF treatment. In order to prepare SmF treatments, the precise amounts of SB and filter cake were mixed with 2.4 and 0.8 kg DM and then, poured into 60 L barrels. The TS values in both SSF and SmF were set to 30 and 10%, respectively. Followed by loading the mixtures into the barrels, the SB was mixed thoroughly with microbial

Table 2. The treatments and their C/N ratio in SSF and SmF cultivations.

Treatments	Abbreviation	C/N ratio
Bagasse	B	94.2
Bagasse + Filter cake	BF	72.2
Bagasse + Filter cake + Urea	BFU	32.5
Bagasse + Filter cake + Activated sludge + Urea	BFAU	32.5
Bagasse + Filter cake + cow Manure + Urea	BFMU	32.5
Bagasse + Filter cake + Compost + Urea	BFCU	32.5
Bagasse + Filter cake + Activated sludge + cow Manure + Compost + Urea	BFAMCU	32.5
Bagasse + Filter cake + Fungi booster + Urea	BFFU	32.5
Bagasse + Filter cake + Poultry manure	BFP	33.5
Bagasse + Filter cake + Activated sludge + Poultry manure	BFAP	32.5
Bagasse + Filter cake + cow Manure + Poultry manure	BFMP	32.5
Bagasse + Filter cake + Compost + Poultry manure	BFCP	32.5
Bagasse + Filter cake + Activated sludge + cow Manure + Compost + Poultry manure	BFAMCP	32.5
Bagasse + Filter cake + Fungi booster + Poultry manure	BFFP	32.5

sources (nitrogen source) and moistened with water. To prepare the microbial inoculation, 5% w/w of the microbial source (such as compost) was agitated at 150 rpm and room temperature for two hours. Then, after settling the solids, the supernatant was added to the relevant treatment.

2.3. SSF setup experiments

The moisture content of the samples was adjusted to 70% over the whole period since the process was aerobic, and the holes in the wall and floor of the barrels allowed air penetration and inhibition of extra water accumulation. Aeration and humidification were carried out regularly every three days for the first two weeks and then, every seven days until the end of the process (75 days), thus providing important factors namely water, airflow, and homogenization for better microorganism performance. In the aeration process, the lids of the barrels are closed and then, rolled on the ground and stirred with a fork. During the process, an adequate amount of water is added to the barrels to keep humidity at 70%.

2.4. SmF setup experiments

Upon combining SB and filter cake with water, they were completely stirred and the weighed values of the microbial inoculation were added to the relevant treatment. In this step, the needed aeration and heat were provided through air pumps and water heaters. The obtained mix was continuously stirred every eight hours using both permanent bubbling and mechanical methods until the end of the process (36 days). Manual mixing can improve the homogenization of the samples and prevent settling and floating of solid particles. The input capacity of the air for each sample was set at 8 L/min at a fixed temperature of 30°C.

2.5. Measurement and analytical methods

The pH values of the sample were measured using a metrohm pH meter device with 1:10 dilution for SSF and without dilution in SmF samples. Each solution was filtered using a Whatman filter paper no. 1 and then, pH turned red in the supernatant. By using an electrical furnace device, DM was measured at 105°C for 72 hours and the weight stabilization for the dried samples was implemented using the following formula [18]:

$$DM = \frac{C - A}{B - A} \times 100, \quad (2)$$

where A is the weight of the empty envelope (or petri dish); B the weight of the envelope (or petri dish) with the sample; and C the weight of the envelope (or petri dish) along with the dried sample at 105°C.

Organic Matter (OM) was determined by burning the sample of dried OM at 550°C in an electric oven using the following formula [18]:

$$OM = \frac{D - A}{C - A} \times 100, \quad (3)$$

where A is the empty cruise weight; C cruise weight with the dried sample at 105°C; and D cruise weight with the dry sample at 550°C.

The Kjeldahl method was employed to determine Total Nitrogen (TN) content of the samples [18]. The Chemical Oxygen Demand (COD) was measured using the closed reflux colorimetric method [18]. The Most Probable Number (MPN) measurements were performed based on the method given in [19]. Infrared spectroscopy analysis was employed in conjunction with a Fourier Transform Infrared (FTIR) Spectroscopy (model WQF-510), which was set to a range of 400 to 4000 cm^{-1} . The dry extracted sample was mixed with KBr and used for FTIR analysis [20].

2.6. Extraction of HA and FA

Extraction of HA and FA was carried out by modified alkaline extraction method in [21]. In this method, 10 g of the sample was stirred with 100 ml of 2 M NaOH (pH = 13) solution for 48 h and then, centrifuged (20 min, 4000 rpm). This stage is useful to separate the sediments from HA and FA. At the next step, HA was separated from FA by adding 3 M HCl to the supernatant and mixed well until it reached pH = 2. Next, the mixture was kept in the dark for 48 h. Finally, HA was separated completely from FA by centrifugation (20 min, 4000 rpm). The HA precipitates were characterized by brown color, while the FA supernatant was characterized by its golden dye. After separating the two phases, both solutions were dried and weighed in an electric oven at 50°C [21].

2.7. Fungal booster preparation and molecular identification

Soil samples were collected from rice fields in Gilan province in the north of Iran. Davet's specific culture medium was used to isolate *Trichoderma* strains [22]. Initially, soil samples were dissolved in 0.04 w/v citric acid solution and moistened. Then, the moistened soil samples were kept at 25°C and shaken at 200 rpm for 24 h. In the subsequent stage, each sample was added to a petri dish in sterile conditions, followed by 2% w/v water agar addition. The petri dish was kept in an incubator at 25°C for 24 h and then, the petri dishes were transferred to Davet medium. Fungi purification was performed by transferring a single hyphal tip grown from a water agar medium onto a potato dextrose agar. The purified isolates were stored in slants containing Potato Dextrose Agar medium (PDA) at 4°C for future use.

2.7.1. Whole DNA extraction and PCR amplification

The isolated fungi strains used as a fungal booster in this study included G57, G139-2, and G130-4, which were identified as '*Trichoderma*' based on morpholog-

ical characteristics and molecular analysis. In order to identify *Trichoderma* isolates using the molecular method, the whole genomic Deoxyribo Nucleic Acid (DNA) was extracted according to the protocol described by Zhong and Steffenson [23]. The Polymerase Chain Reaction (PCR) amplification was conducted for amplification of ITS-rDNA region in a 20 μ l reaction volume containing 0.2 unit/ μ l *Taq* DNA polymerase (Ampliqon, Denmark); 1.5 mM MgCl₂; 0.2 μ M of ITS-1 (5'-CGTAGGTGAACCTGCGG-3') and ITS-4 primer (5'-TCCTCCGCTTATTGATATGC-3') as well as 10 ng genomic extracted DNA. DNA concentration and purity were analyzed by spectrophotometry using 260 and 280 nm absorbance values. To confirm DNA amplification, 1 μ l of each amplified product was electrophoresed with 1% agarose gels and visualized under UV light using the GelDoc-It System. Amplified products were separated by agarose gel electrophoresis and aligned using MEGA 6.06. Then, sequence analysis was performed using TrichoBlast software. All identified species were deposited in the culture collection of the Agriculture Biotechnology Research Institute of Iran (ABRII CC). Blast analysis revealed that selected strains G57, G139-2, and G130-4 had a 99% similarity to *T. harzianum*. For the inoculation of fungal boosters, after 72 h of culture, biomass was shaved from the surface of the PDA medium (from each of the three fungal strains) and added to the relevant treatment at a rate of 1 cm²/L.

2.8. Pilot plant setup

The main purpose of running the pilot plant setup was to replicate the best selected treatment of 60 L scale experiments (BFFU treatment) on a larger scale. The second pilot setup capacity was a 1000-liter CSTR fermenter with a Rushton impeller running at 30 rpm and temperature of 30°C by a jacket heater. The input capacity of air was set to 93 L/min by an air pump. The implementation of the pilot phase lasted 36 days. The number of compounds loaded into the pilot container consisted of 75 kg of SB, 25 kg of filter cake (ratio 3:1), and 300 g of urea (dissolved in water before adding to the mixture), which were mixed thoroughly by water addition and fed with the fungal booster. The total volume reached 700 liters plus water (TS = 10%).

2.9. Statistical analysis

Statistical analysis was performed using the one-way ANOVA statistical method (SPSS software version 19) to determine the effectiveness of each factor and their interaction. Significance level was considered using the *p*-value factor, and values of *p* ≤ 0.05 were assumed to be statistically significant. Data matching was also performed using Duncan's method. A Duncan's test (post-hoc test) was employed to achieve significant results. Moreover, it is necessary to

compare mean differences between individual data and different groups in order to determine the origin of the differences. Homology of variance (Levene's test) was also performed (StatSoft, Tulsa, OK) as a robust test to assess the quality of homogeneity among statistical data obtained from experiments. The significance level (under 0.05) suggests that the assumption of equal variances is refracted [24].

3. Results and discussion

Several key parameters including temperature, pH and, C/N ratio are directly affected by the decomposition of organic material via dominant microorganisms in each stage of the composting process. According to the published papers, these microorganisms comprise a wide variety of mesophyll and thermophile fungi and bacteria species, e.g., *Trichoderma*, actinomycete, *Aspergillus*, *Penicillium*, *Nocardia*, *Pseudomonas*, *Bacillus*, etc. [25]. Hence, it is vital to monitor these physicochemical parameters during the entire four-stage composting process to ensure the performance of microorganisms and the accuracy of the process in progression [26]. In this section, the detailed data collected during the process are discussed (TMECC 229 2002).

3.1. SSF experiments

The SSF is a suitable culturing method of fermentation practical in many biodegradation processes and production of various biotechnological products such as compost, enzymes, edible fungi, etc. [27,28]. In composting, the purpose of SSF culture is to decompose organic wastes and achieve a non-biodegradable final compound called humus. The decomposition conditions in SSF have a critical effect on HA and FA production. Time of beginning the thermophilic phase and quality and quantity of biodegradation during the process are the parameters that significantly affect the final compounds [29].

3.1.1. Monitoring pH during the SSF process

One of the important parameters in biological processes is the pH of the culture media and it indicates the acidity rate of the compost material. In fact, the pH digits increase regularly as the process progresses due to the diminishing activity of compost microorganisms whose best decomposition function is in neutral and acidic conditions. At the end of the process, pH begins to increase. This pH rise is directly related to the decreasing number of active microorganisms at high pH and high production of ammonia (ammonification) during the cooling stage [30]. In other words, in the first days of the process, pH was lower (6 to 8 in different treatments) since the organic acids were released for microorganisms to consume. Then, the pH increased over time due to the consumption of

organic acids and stabilized the optimum range for the microorganisms involved. In SSF processes of organic compounds (such as a composting process), pH is a sign that the decomposition process is doing well. The pH value of all treatments followed a negligible upward trend over a 36-day period.

3.1.2. Monitoring temperature during the SSF process

One of the other decisive factors in SSF processes, such as composting, is temperature. The rapid increase in temperature in the composted SSF indicates a higher rate of decomposition and further microorganism activities [31]. Compost piles consist of three predominant categories of bacterial species (psychrophiles, mesophiles, and thermophiles) that are active in four different stages of the composting process. High breakdown of organic compounds via fast-growing bacterial species to obtain energy increases the heat arising from the composting process, which entails the progress and prolongation of the thermophilic stage and rise in the temperature. After the aforementioned productive stage, substrates are consumed and the temperatures begin to cool [32]. Figure 1 illustrates that all treatments began at 20°C and then, the temperature reached a peak between the second and fifth weeks. After 42 days, a steady decline over the process was observed due to the reduction of simple decomposing compounds in the decomposing biomass. One significant point worth mentioning is that temperature in samples without microbial inoculation was considerably low; therefore, low decomposition occurred in these treatments. Hence, it is concluded that temperature is a key factor in better decomposition. Moreover, the addition of microbial inoculants such as compost, cow manure, and fungi booster contributed greatly to reaching the highest temperature at around 60°C. The treatments that had been incorporated into the thermophilic phase earlier than the other treatments included BFMU, BFCU, and BFFU with temperatures of 53, 54, and 54°C, respectively, as seen in Figure 1.

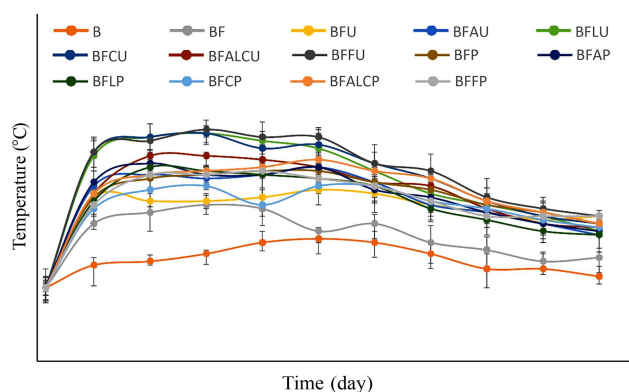


Figure 1. Temperature changes in SSF treatments over time.

3.1.3. Monitoring C/N ratio in different SSF treatments

In SSF treatments, variation in OC value is one of the best parameters that indicates the rate of decomposition [33]. Generally, it is expected that gradual OC decomposition could lead to weight loss and increase in the total nitrogen content. Admittedly, the initial ratio of C/N plays a crucial role in the accuracy of composting and decomposition stages of OC because it acts as an indicator of nutrient balance for compost microorganisms [26]. The optimal C/N ratio for the growth of microorganisms ranges from 30 to 35 [34], which was regulated at 32.5 at the beginning of the process (except B and BF treatments as controls). The value of OC during the SSF process in the treatments was measured, as presented in Figure 2. Based on the results, the highest rate of decomposition during the process belongs to the BFFU and BFFP treatments with 42.9 and 43.1% reductions of OC, respectively, as shown in Figure 2. According to the obtained results, the presence of fungal booster had a high impact on the removal of OC. During the biomass decomposition, the C/N ratio decreased gradually and leveled off for the final product. The C/N ratio was measured at the end of the process. As can be seen in the results represented in Table 2, the C/N ratio in the B treatment at the end of the process was 113.5. This value indicates incomplete decomposition due to the initial C/N ratio (94.2). Furthermore, in case of BF treatment, this ratio stood at 81.8, which indicates incomplete decomposition due to the lack of adjustment of the initial C/N ratio (72.2). In the BFU treatment, the initial C/N ratio was regulated at 32.5; however, due to the lack of microbial inoculation, the decomposition of this treatment was not complete. In other treatments, the final C/N ratio was acceptable at the stabilization point. The best results of C/N point to the values of 15.3, 15.8, 15.3, and 15.9 for BFMU, BFAMCU, BFFU, and BFFP treatments, respectively, as shown in Table 3.

3.2. SmF experiments

Another culture method that enjoys high efficiency in

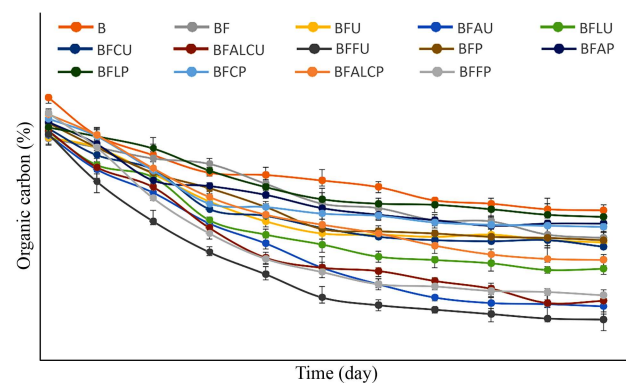


Figure 2. The OC changes in SSF treatments over time.

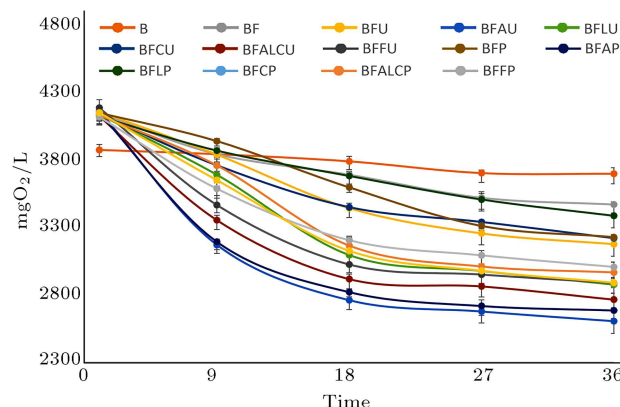
Table 3. C/N ratio in different SSF treatments at the end of the process.

Treatment	C (%)	N (%)	C/N ratio
B	29.5 ± 0.3	0.26 ± 0.1	113.5
BF	27.0 ± 0.5	0.33 ± 0.2	81.8
BFU	26.6 ± 0.4	0.65 ± 0.1	40.9
BFAU	20.8 ± 0.1	1.08 ± 0.2	19.3
BFMU	24.2 ± 0.9	1.58 ± 0.1	15.3
BFCU	26.2 ± 0.7	1.38 ± 0.1	19.0
BFAMCU	21.3 ± 0.7	1.35 ± 0.2	15.8
BFFU	19.6 ± 0.4	1.28 ± 0.3	15.3
BFP	26.8 ± 0.2	0.64 ± 0.1	41.9
BFAP	28.3 ± 0.6	1.19 ± 0.2	23.8
BFMP	28.9 ± 0.3	1.48 ± 0.1	19.5
BFCP	28.3 ± 0.1	1.40 ± 0.4	20.2
BFAMCP	25.0 ± 0.5	1.39 ± 0.1	18.0
BFFP	21.8 ± 0.4	1.37 ± 0.2	15.9

bio-production and widespread use, especially in microbial biotechnology, is SmF. Many biotechnological and microbial products are produced via this type of culture process [35]. Therefore, SmF is used to produce HA and FA in this study. It should be mentioned that this is the first report on production of HA and FA from agricultural wastes by SmF method.

3.2.1. Monitoring pH during the SmF

In biological processes, especially in the case of liquid culture media application, pH changes represent a good indicator determining whether the whole process is well organized or not [36]. It was found that all samples exhibited a steady rise in pH from 6 to 8 in the first 9 days; then, the first half of the samples fell gradually, as opposed to the rest of the samples that experienced a slight upward trend over 9–27 days. Finally, in the last cycle, all the samples tended to a pH of around 7. The pH of all treatments reached just over 7 at the end of the process. During the whole process, pH values underwent some fluctuations. The reason for pH changes during the process is the high availability of organic acids at the beginning of the process, which are gradually consumed by predominant microorganisms, especially fungal species. It is worth mentioning that neutral to acidic pH ranges provide the best condition for fungal species to decompose lignin and cellulose. However, as the process enters the next stages, neutralization of organic acids begins and these species lose their activity and start to diminish. In such circumstances, other microorganisms begin waste consumption and a high rate of ammonification gives rise to a range of high pHs [30]. In samples with a high pH, organic acids were produced rapidly and consumed by the microorganisms. In contrast, treatments with

**Figure 3.** Changes in COD of treatments in the SmF process over time.

a decreasing trend produced stable organic acids in the solution. Among the treatments, the highest and lowest pHs at the end of the process belonged to BFAU and BFU with values of 7.45 and 7.01, respectively.

3.2.2. Monitoring COD during the SmF

COD measurement is a proper indicator of decomposition and a better tool in liquid cultures than the C/N ratio in solid conditions [37,38]. COD reduction is a sign of decomposition of organic compounds through microorganisms that consume available oxygen in the fermentation media [39]. The COD values of different SmF treatments over time are given in Figure 3. The initial COD values for all treatments (except “bagasse” as control) ranged from 4120 to 4187 mg O₂/L, as shown in Figure 3. The amount of COD in all of the samples was reduced during the process. In all of the samples (except B and BF), the observed COD declined dramatically from above 4000 to around 2700–3600 mg O₂/L in different treatments after 18 days and then, the graphs show a slight decline for the rest of the process. The highest and lowest COD removal rates during the process related to BFAU and B were 37.4 and 4.5% in 36 days, as given in Figure 3.

3.3. Comparison of produced HA and FA in SSF and SmF

The best treatments were selected in terms of HA and FA production. Therefore, the main indicator for choosing the best method was the production rate of these two acids. The values of extracted HA and FA is shown in Table 4. The amount of produced HA in SSF treatments was in the range of 5.2 to 39.6 g/kg. The detailed production amounts using SmF treatments increased from 12.6 to 128.4 g/kg and from 1.2 to 12 g/L, respectively. FA production rates via SSF treatment ranged from 3.1 to 23.6 g/kg. FA production via SmF treatment (ranged from 7.3 to 79.1 g/kg and 0.7 to 7.5 g/L) was extracted, as seen in Table 4. In terms of production efficiency via SSF, the lowest and the highest digits were 1.1 and

Table 4. The values of extracted HA and FA in SSF and SmF.

Parameter	HA	HA	HA	HA		FA	FA	FA	FA	
	Value	efficiency	Value	efficiency	Value	efficiency	Value	efficiency	Value	efficiency
Fermentation	SSF	SSF	SmF	SSF	SmF	SSF	SSF	SmF	SSF	SmF
Unit	g/kg DW	% OM	g/kg DW	g/L	% OM	g/kg DW	% OM	g/kg DW	g/L	% OM
B	5.2 ± 0.2	1.1 ± 0.3	12.6 ± 0.6	1.2 ± 0.1	3.1 ± 0.5	3.1 ± 0.3	0.8 ± 0.2	7.3 ± 0.2	0.7 ± 0.0	1.8 ± 0.1
BF	8.4 ± 0.5	2.3 ± 0.1	22.9 ± 0.4	2.1 ± 0.2	6.0 ± 0.4	4.9 ± 0.1	1.4 ± 0.3	12.7 ± 1.0	1.2 ± 0.1	3.5 ± 0.4
BFU	18.7 ± 0.1	5.2 ± 0.4	38.5 ± 0.3	3.6 ± 0.4	10.7 ± 0.2	11.7 ± 0.3	3.2 ± 0.1	21.6 ± 0.1	2.0 ± 0.3	6.0 ± 0.2
BFAU	26.4 ± 0.4	7.1 ± 0.2	85.9 ± 0.3	8.0 ± 0.7	23.7 ± 1.3	15.5 ± 0.4	4.3 ± 0.2	33.5 ± 0.7	3.2 ± 0.1	9.2 ± 0.1
BFMU	31.1 ± 0.3	8.3 ± 0.7	109.1 ± 0.1	10.2 ± 0.1	29.8 ± 0.1	18.7 ± 0.4	5.1 ± 0.2	67.8 ± 0.3	6.5 ± 0.3	18.5 ± 0.3
BFCU	20.5 ± 0.4	5.6 ± 0.3	79.3 ± 0.8	7.4 ± 0.5	21.5 ± 0.5	11.2 ± 0.7	3.0 ± 0.1	46.0 ± 0.4	4.4 ± 0.2	12.5 ± 0.9
BFAMCU	32.3 ± 0.8	9.8 ± 0.6	80.2 ± 0.4	7.5 ± 0.2	21.9 ± 0.9	18.9 ± 1.1	5.2 ± 0.2	49.3 ± 0.1	4.7 ± 0.4	13.4 ± 0.2
BFFU	39.6 ± 0.1	10.9 ± 0.1	128.4 ± 1.6	12.0 ± 0.8	35.3 ± 0.4	23.6 ± 0.3	6.4 ± 0.3	79.1 ± 1.5	7.5 ± 0.6	21.7 ± 0.8
BFFP	19.9 ± 1.1	4.8 ± 0.6	42.6 ± 0.4	4.0 ± 0.4	11.4 ± 0.8	10.7 ± 0.1	2.9 ± 0.0	25.4 ± 0.8	2.4 ± 0.1	7.0 ± 0.3
BFAP	26.8 ± 0.4	7.0 ± 0.2	58.8 ± 1.3	5.5 ± 0.5	15.7 ± 0.1	15.0 ± 0.8	4.0 ± 0.4	16.2 ± 0.3	1.5 ± 0.0	4.3 ± 0.2
BFMP	32.4 ± 0.3	8.9 ± 0.4	96.5 ± 0.4	9.0 ± 0.3	26.1 ± 0.1	19.1 ± 0.3	5.2 ± 0.3	57.3 ± 0.8	5.4 ± 0.2	15.3 ± 1.3
BFCP	22.1 ± 0.1	6.2 ± 0.3	76.8 ± 1.2	7.2 ± 0.1	20.3 ± 0.2	12.4 ± 0.9	3.3 ± 0.2	41.8 ± 0.1	4.0 ± 0.4	11.1 ± 0.1
BFAMCP	34.4 ± 0.8	9.3 ± 0.1	40.0 ± 0.3	3.8 ± 0.3	10.5 ± 1.0	18.3 ± 0.4	4.8 ± 0.1	22.5 ± 0.4	2.1 ± 0.3	5.9 ± 0.0
BFFP	38.8 ± 0.3	10.2 ± 0.9	38.5 ± 0.1	3.6 ± 0.1	10.1 ± 0.5	22.9 ± 1.2	5.9 ± 0.4	21.3 ± 0.3	2.0 ± 0.2	5.6 ± 0.3

10.9% HA/OM (Organic Matter) and were 0.8 and 6.4% FA/OM, respectively. The production efficiency in the case of SmF treatments ranged between 3.1 and 35.3% HA/OM and 1.8 and 21.7% FA/OM, as presented in Table 4. In SSF, the best treatments were BFFU and BFFP that produced 39.6 and 38.8 g/kg HA as well as 22.9 and 23.6 g/kg FA, respectively, as seen in Table 4. In SmF, BFFU and BFMU treatments with production values of 128.4 and 109.1 g/kg HA as well as 79.1 and 67.8 g/kg FA were the best treatments, respectively. Having a comprehensive calculation, the total values of HA+FA produced via BFFU and BFMU treatments were the highest percentages of 57.2 and 48.3% (HA+FA)/OM, respectively, as seen in Table 4. This means 57.2 and 48.3% of the total OM converted into these two valuable compounds. Other measured parameters including temperature, OC, COD, pH, and C/N ratio values during the process were in line with the amount of extracted HA and FA. Comparison of SSF and SmF treatments clearly showed that SmF culture achieved much better results than SSF type due to the better conditions in place for the growth of microorganisms, microbial activity in the presence of significant water volume, homogenization via better mixing, and aeration in the SmF method [35]. According to the obtained results and higher production of HA and FA, BFFU treatment was selected to go through more experiments including running advanced-scale and pilot-scale setups. Of note, HA and FA production values determined using the method proposed in this study, i.e., SmF method, were quite significant and also, the values obtained in this research were higher than other HA reports from SB or other agricultural wastes. This indicates the appropriate choice of culture

method, the appropriate microbial booster, and other items applied in the treatments such as appropriate concentration of compounds during the process.

3.4. Fungal booster preparation and molecular identification

Trichoderma species are widely distributed filamentous fungi in various environmental conditions whose potential in industrial-scale production of cellulase and hemicelluloses enzymes is well proved. These remarkable features have made the *Trichoderma* genus an efficient and well-known enzyme producer in industrial biotechnology projects. Regarding the highly complex structure of HA and its resistance to microbial degradation, a cocktail of three *Trichoderma* spp. was prepared and inoculated to the relevant treatment at a rate of 1 cm²/L [40].

3.4.1. PCR amplification

A product of approximately 600 bp was obtained from all the amplifications with primers ITS1 and ITS2 for three selected *Trichoderma* isolates (G57, G139-2, and, G130-4), as shown in Figure 4.

3.4.2. *Trichoderma* species identification by bioinformatics analysis

Bioinformatics tools were applied to species identification. TrichOKEY2 (<https://www.trichokey.com/index.php/trichomarka>) [41] and TrichoBLAST (isth.info/tools/blast/blast.php) [42] were used for analyzing sequences of ITS 1 and ITS 2 and alignment determined using MEGA 6.06 software. Based on ITS1 and ITS 2, species complexes were identified as *T. harzianum*. For taxonomic identification, sequences were compared with publicly available sequences de-

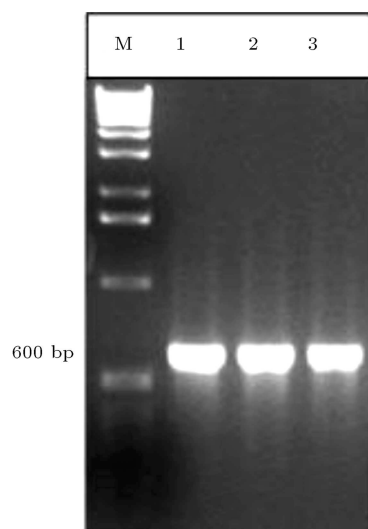


Figure 4. Amplification of ITS1 and ITS2 DNA fragments in the selected *Trichoderma* isolates. M: DNA ladder, Mix 1: G57, 2: G139-2, 3: G130-4.

posited at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) by using a Basic Local Alignment Search Tool (BLAST). The most conclusive identification obtained TrichoMARK selection using NCBI GenBank BLAST search. NCBI GenBank BLAST analysis of all the investigated loci fulfilled identifications. Nucleotide Blast search of 1, 2 and, 3 strains (GenBank accession no.: KM456214, KM456216 and, KM456217) revealed 99% similarity to strains of *T. harzianum* (Figure 5). All identified species were deposited in the culture collection of the (ABRII CC) and NCBI.

3.5. Pilot-scale results

Based on the results obtained from the first round of experiments, the most successful treatment used at the pilot phase was BFFU. The pilot phase lasted for over a period of 36 days and the parameters were measured during the period given in Table 5. As shown in the results, the values of HA and FA production are slightly improved. According to the obtained results of SmF designed experiments, the production of HA and FA in the selected treatment was 128.4 and 79.1

g/kg, respectively. While production values of HA and FA at the pilot phase were slightly higher at 142.5 and 83.9 g/kg, respectively, as given in Table 5. Better results were achieved in the pilot setup due to better and continuous mechanical agitation at this phase [43]. Mechanical agitation led to complete homogenization, hence better accessibility of undegraded organic compounds for microorganisms [44]. There was a dramatic decrease in COD values from 4210 to 2762 mg O₂/L at the end of the process (34.4% COD removal). The pH was almost neutral during the process. TS rose from 10% at the beginning to 6.6% at the end of the process due to the decomposition of organic compounds and conversion into various volatile gases, e.g., CO₂ and H₂O [45]. The number of fungi colonies measured over time rose significantly from 10 to 300 MPN/ml (Table 5). The number of bacteria in the first week was more than other measured intervals due to the gradual growth rise of fungal masses during the process and the promising possibility of producing inhibiting compounds against bacterial growth. The production of high-value chemicals in microbial biotechnology encounters several obstacles, which are mainly related to the difficulty in biodegradation of lignocellulos compounds (such as SB), low production yield, laborious and time-consuming process, and high production costs [14,27,28,35]. Therefore, finding a solution that can increase production yield and cut the final cost and time is precious. Among these problems, the production of these two organic acids in an industrial form is currently done by extraction from specific soils such as Leonhardtite HAs [46,47]. Admittedly, producing HA and FA from waste is, industrially, much more justifiable, especially if the proposed process cuts the price and duration of the production process [48]. Therefore, this study attempted to rise the degradation of SB lignocellulos structure. In doing so, there are two decisive factors in the design of the experiments: the microbial source and the prepared culture. Microbial sources such as activated sludge, compost, and manure are rich in various types of microorganisms and are assumed to be a suitable microbial mixture for the biodegradation of lignocellulose compounds. A fungi

Table 5. The results of the pilot-scale experiment related to BFFU treatment.

Time day	FA g/L	FA g/kg DM	FA % OM	HA g/L	HA g/kg DM	HA % OM	TS %	COD mg O ₂ /L	pH	Bacteria MPN/ml	Fungi MPN/ml
1	0.1 ± 0.0	0.7 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	2.2 ± 0.3	0.6 ± 0.3	10.0 ± 0.7	4210 ± 62	7.4 ± 0.2	10 ²	10
5	1.7 ± 0.2	18.3 ± 0.5	5.0 ± 0.3	2.8 ± 0.1	29.6 ± 1.1	8.1 ± 0.5	9.8 ± 0.6	3911 ± 45	6.9 ± 0.4	2 × 10 ³	200
10	2.8 ± 0.1	29.6 ± 1.0	8.1 ± 0.6	4.9 ± 0.2	51.7 ± 2.2	14.2 ± 0.7	9.1 ± 0.5	3642 ± 38	6.6 ± 0.2	2 × 10 ³	300
15	5.0 ± 0.2	52.0 ± 2.1	14.3 ± 0.4	8.3 ± 0.3	87.4 ± 3.3	24.0 ± 0.4	8.3 ± 0.6	3255 ± 29	6.4 ± 0.2	3 × 10 ³	300
20	6.1 ± 0.3	63.9 ± 3.0	17.5 ± 0.9	10.2 ± 0.4	107.3 ± 4.3	29.5 ± 1.4	7.7 ± 0.2	3090 ± 22	6.4 ± 0.3	3 × 10 ³	200
25	7.4 ± 0.1	77.2 ± 1.1	21.2 ± 0.5	12.6 ± 0.4	132.2 ± 4.5	36.3 ± 2.3	6.8 ± 0.4	2983 ± 30	6.4 ± 0.4	2 × 10 ³	300
30	8.0 ± 0.2	83.8 ± 2.0	23.0 ± 0.8	13.5 ± 0.2	141.3 ± 2.3	38.8 ± 1.1	6.8 ± 0.5	2835 ± 17	7.0 ± 0.1	2 × 10 ³	200
35	8.0 ± 0.3	83.9 ± 3.2	23.1 ± 0.7	13.60 ± .3	142.5 ± 3.4	39.1 ± 1.3	6.6 ± 0.3	2762 ± 25	7.1 ± 0.1	2 × 10 ³	300

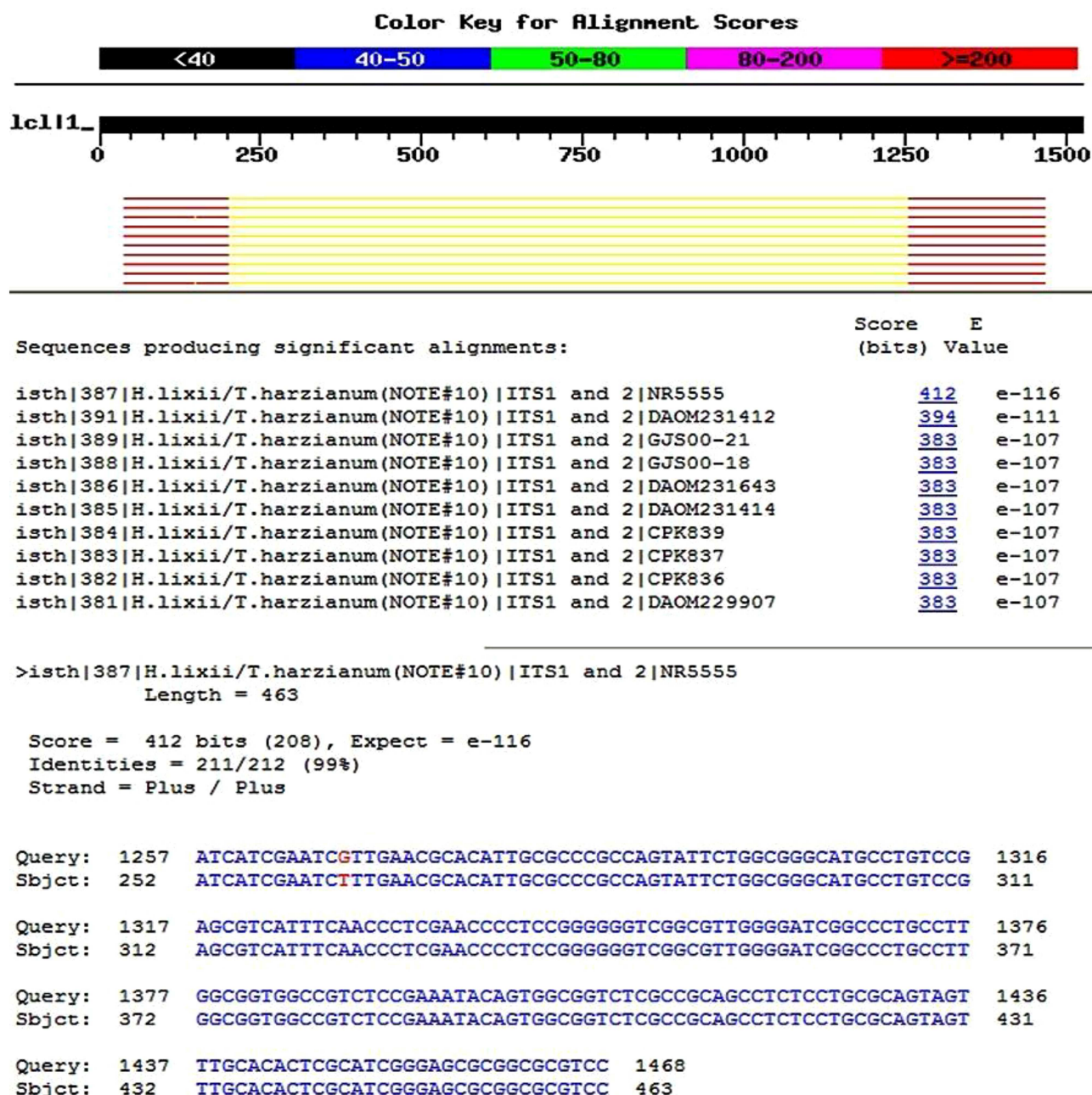


Figure 5. The G57 strain showed a 99% similarity to strains of *T. harzianum* (Accession No. NR5555).

genus such as *Trichoderma* has a very strong and diverse enzyme system that breaks down the structure of the lignocellulose compounds used in the present study [43]. Cultivation type is another important factor in the production of biotechnological products from biomass. Compared with SSF, SmF enjoys high homogeneity, complete mixing of the mixtures, and availability of water to microorganisms [35]. In order to compare the results of these two types of culture in the production of HA and FA, both were used in the preset research. The results indicate that the SmF obtained much better yield in a shorter time than SSF culture. Production values in the best treatment

of SmF during 35 days were 35.3 and 39.1 for HA and 21.7 and 13.6 for FA on 60 and 1000 liter scales, respectively. Table 1 summarizes the results of other research studies conducted to produce HA and FA from different substrates. As introduced in Table 1, HA is produced from various sources of biomass. It is noteworthy that the production method is composting in all studies, which is one of SSF culture types. The SSF cultivation method is a suitable and cost-effective method that is viably applicable to agricultural lands and production of organic fertilizer [35]. However, if the purpose of organic decomposition is to achieve higher amounts of HA and FA for different purposes such as

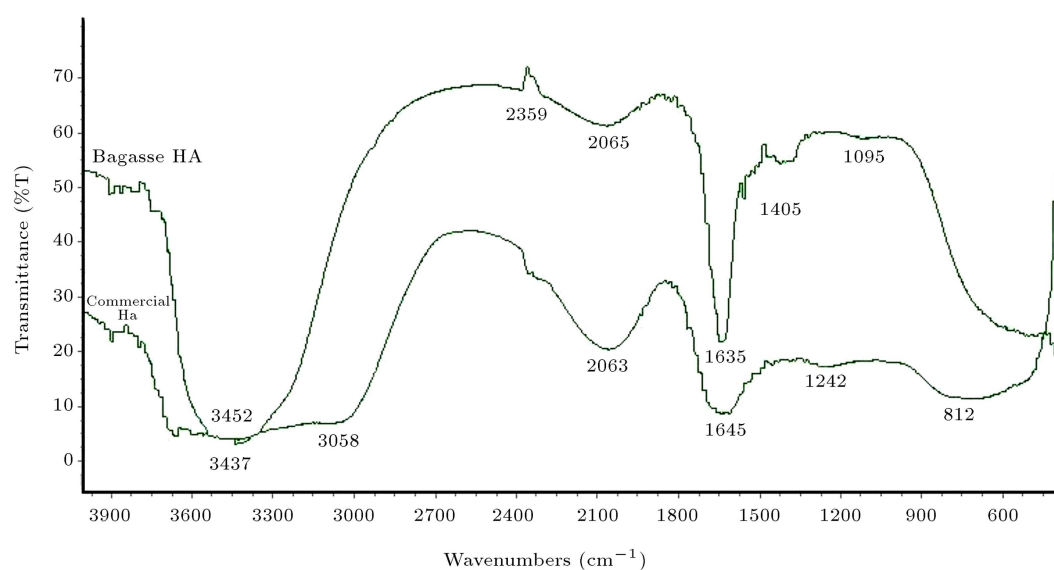


Figure 6. FTIR analysis spectrum of the HA extracted from SB and CS.

extraction and commercialization, it is very important to choose a method able to produce higher amounts. Production of HA from SB has been reported in various studies [49]. In a comprehensive study conducted by our research team, there was no report on the use of SmF to produce HA and FA. The present study is the first report in this regard. Another important point is to compare the production amounts that were presented by different reports. In most reports, the amount of production is much lower than that obtained in the present research. Authors in [50] determined that the total produced HA from composted pineapple leaf residue was 20% DM, as shown in Table 1. It is believed that the leaves of plants, unlike SB, have an easy degradable structure for the composting process.

3.6. Structural analysis

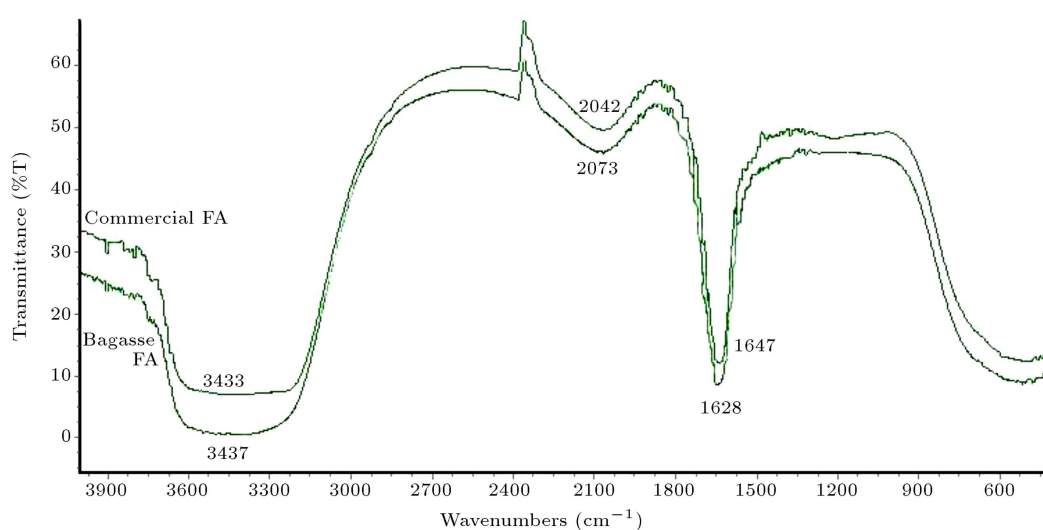
Lignocellulose compounds such as HA and FA are known for their highly complex chemical structure that have made it impossible to accurately determine the structural composition. Various powerful tools that have been applied to characterize these compound structures include nuclear magnetic resonance spectrometry, gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry, chromatography, etc. There is no effective method to fully identify HA and FA structures. However, some methods (NMR and FTIR) have been used to address this issue so far [51]. In this study, we applied FTIR spectra as a qualitative method for monitoring functional groups in HA and FA and made a comparison with the Commercial Sample (CS). According to the FTIR spectrum given in Figure 6, the sample of HA extracted from SB revealed peaks at 3437, 2065, and 1635 cm^{-1} . In addition, in the CS, the peaks were observed at

3452, 2359, and 1645 cm^{-1} , as given in Figure 6. The presence of sharp peaks in the range of 1600 to 1700 and 3400 to 3500 indicates many similarities between the HA structures of SB and CS. In the case of FA extracted from SB, sharp peaks were observed at 3437, 2073, and 1628 cm^{-1} . In addition, CS's FA had sharp peaks at 3433, 2042, and 1647 cm^{-1} in the FTIR spectrum, as given in Figure 6. The sharp peaks in both spectra ranged from 3430 to 3440 cm^{-1} and from 2040 to 2080 cm^{-1} as well as 1620 to 1650 cm^{-1} . Figure 6 shows many structural similarities between these two compounds. The peaks in the spectra had different functional groups in the structures of HA and FA, which are described below. The different intervals in the FTIR spectrum represent different functional groups and express structural features. The intervals and related functional groups are introduced in Table 6. Regarding the analysis performed on the extracted HA of SB and CS, the peaks were seen at 3452 and 3437 cm^{-1} . Figure 6 presents the presence of hydroxyl and N-H stretching of amine and amide groups in the structure of both two compounds, SB and CS [52]. The peaks at 1635 and 1645 cm^{-1} (Figure 6) indicate the presence of oxygenated groups of CO and COO^- , representing the bond of the aromatic band (amide I) [53]. The peaks at 1405 and 1242 cm^{-1} (Figure 6) belonged to the hydroxyl group (such as phenols) and aliphatic C-H stretching of carbohydrates [20]. Aromatic groups also appear at a peak of 800 cm^{-1} (Figure 6) [52]. As shown in the graph of the FA extracted from SB and CS, peaks at 3433 and 3437 cm^{-1} (Figure 7) can be assigned to the hydroxyl and N-H groups in the structure of these compounds [52]. In addition, 1647 and 1628 cm^{-1} peaks were attributed to the groups containing C=O, C=C functional groups (Figure 7) [20]. From the analysis spectrum, there

Table 6. Functional groups detected and compared in HA and FA from SB and CS.

Wave number cm^{-1}	Functional groups	Peak intensity of HA		Peak intensity of FA	
		SB	CS	SB	CS
3000–3700	OH	+++	+++	+++	+++
3000–3100	Aliphatic C–H	-	+	-	-
1540–1870	C=O, C=C	+++	+++	+++	+++
1350–1450	Aliphatic C–H	+	-	-	-
1000–1300	COOH	-	+	-	-
700–900	Aromatic	++	+++	++	++

Note: +++: High strong; ++: Strong; +: Weak; -: Not exist

**Figure 7.** FTIR analysis spectrum of the FA extracted from SB and CS.

are some differences between HAs extracted from SB and CS samples. Despite some differences in the FTIR peaks obtained from SB and CS, many structural similarities were observed between both extracted HA and FA. These differences could result from the pellet drying time that would affect the classification of different functional groups. Moreover, the main goal of using qualitative tools like FTIR is to compare specific peaks dedicated to a particular functional group. However, the appearance of differences in the functional regions could facilitate the identification of HA and FA structures. Of note, structural similarities observed in FA were more than HA comparing SB and CS.

4. Conclusion

SmF is the most popular and widely employed technique in the industrial-scale production of commercial products, e.g., various secondary metabolites, acetic acid, ethanol, single-cell protein, and different enzymes. The reason for this preference is that liquid culture provides strict and easy control on fermentation parameters (temperature, moisture, pH, oxygen transfer, and

aeration) and microorganism selection based on needed nutrients. In addition, the liquid medium provides a suitable condition for heat, microorganisms, and mass transfer. In addition, higher yields, easy purification in downstream steps, lower costs in applied devices, labor section and environmental contamination are worth mentioning. In this study, SmF achieved better yield in the production of HA and FA from SB (or likely other agricultural wastes) due to the abovementioned positive points such as reduction of the whole process time and total expenses and greater control on various parameters. SmF culture method has the potential to degrade hard substrates such as SB, which can be used as a low-cost and environmentally-friendly substrate for production of different valuable compounds. In this way, HA could be used for accelerating crop growth. In the present study, 57.2% of the raw SB converted into HA and FA via SmF method. In contrast, HA and FA production via the SSF was difficult to control and low in yield. This high amount of produced HA could pave the way for HA commercialization and guarantee a source of revenue for agro-industries. Furthermore, biomass-derived after HA and FA production has high

quality and could be considered as an effective enhancer for plant growth and soil fertility. Optimization of SSF conditions could be achievable by inoculation of economically feasible enzymes and, also increasing the total process time predicted to positively effect on the final production titer.

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Abbreviations

(ABRII CC)	Agriculture Biotechnology Research Institute of Iran Culture Collection
(BLAST)	Basic Local Alignment Search Tool
(COD)	Chemical Oxygen Demand
(CS)	Commercial Sample
(CSTR)	Continuous Stirred-Tank Reactor
(DNA)	Deoxyribo Nucleic Acid
(DM)	Dry Matter
(FTIR)	Fourier Transforms Infrared Spectroscopy
(FA)	Fulvic Acid
(HA)	Humic Acid
(NCBI)	National Center for Biotechnology Information
(OC)	Organic Carbon
(OM)	Organic Matter
(PCR)	Polymerase Chain Reaction
(PDA)	Potato Dextrose Agar medium
(SSF)	Solid State Fermentation
(SmF)	Submerged Fermentation
(SB)	Sugarcane Bagasse
(TN)	Total Nitrogen

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