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Molecular Profiling of Sugarcane Wine, a Traditional Fermented Beverage (Loungouila) from Madingou

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Abstract

A number of studies reported that traditional fermented beverages possessed pharmaceutical biomolecules involved in biocatalysis for good therapeutic effects on various pathology including tumor, diabetes, inflammation, and obesity. This dimension of understanding is the prerogative of the biomolecular profile found in these fermented foods and beverages. The current work aimed to study the postfermentation molecular profile of the Congolese fermented beverage (Lougwila). The determination of pH, the acidity titratable, the distillation of sugar cane, the determination of total polyphenols and flavonoids and the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) have been used. As results, the fermenting sugar cane juice at room temperature for a period of seven days, leading to a decrease of the pH value to 4.25 ± 0.10 and increase in titratable acidity and alcohol content of at least 6.421 g/L (w/v) and 7% respectively. The polyphenol concentration of Loungouila increase from 14.9 to 20.5 mg Eq AG/g Ms after 10 days of fermentation. The flavonoid concentration varies from 1.70 to 5.22 mg Eq Cat/g Ms. among 46 isolates of *Bacillus* species, 55% (25/45) were able to show a very interesting clear zones in terms of cellulolytic activity with the percentage ranging from 37.3% to 83.9%, and 41.3% of amylolytic activity for the percentage ranging from 52.02% to 75%. 65.21% (30/46) of the isolates tested were found to be positive by the caseinolytic test with zones of inhibition ranging in diameter from 1.10 ± 0.09 to 3.25 ± 0.07 cm. In addition to the determination of biomolecule profile, 34.78% (16/46) of *Bacillus* isolates were able to produce biosurfactants with percentages ranging

from 14% to 100%. Proteomic profiling of Loungouila has been investigated by using MALDI-TOF Technique. Short sequences showed 100% identity and were associated with AprE, SubC, amyE, NprE, CelA, lytF, Mut, and ykfC proteins. The National Center for Biotechnology Information (NCBI) allowed to associate short sequences to *Bacillus* species.

Keywords

Loungouila, Molecular Profiling, Bacillus, Polyphenol, Flovanoids, Biosurfactant

1. Introduction

Sugarcane (*Saccharum officinarum*), a perennial grass of the family Poaceae, primarily cultivated for its juice from which sugar is processed [1]. Most of the world's sugarcane is grown in subtropical and tropical areas including Madingou which is a town located in the southern Republic of the Congo. It is the capital city of the Bouenza Region. In the Republic of Congo, the sugarcane Wine (also called Loungouila or Loungwila or Loung) is produced exclusively in all districts of the Department of Bouenza. The process remains traditional and consists of incubating in anaerobic conditions, in large containers (cans, pots, drums, etc.), the nectar extracted from this plant for 5 to 10 days. This incubation is done systematically in the presence of many additives such as the previously peeled stems of *Costus afer* and crushed corn, in order to accelerate the fermentation process. This drink was also carried to Gabon. It is also called Musungu or Malamba [2]. Sugarcane juice is widely consumed and is an excellent medium for fermentation in the development of alcoholic beverages. Phenolic and volatile compounds have been recently reported in the production of sugarcane vinegar and brown sugarcane [3] [4]. In addition in Brazil sugarcane wine has been particularly and experimentally studied for its composition in volatile compounds [5]. In an elderly population with high cardiovascular risk, the moderate consumption of wine is highly advised in order to reduce lower prevalence of metabolic syndrome. This can modulate human intestinal inflammatory response [6] [7] [8] [9] [10] because of its high content of antioxidants and phenolic compounds. The juice fermentation process is a combination of complex interactions involving a variety of materials. It has been recently shown that fermentation can increase the biomolecule content including phenolic compounds. The genera *Bacillus* are involved in this process since they can secrete several enzymes to increase intracellular bioincrease [11].

The composition and aromas of traditional alcoholic beverages are derived mainly from the raw materials used in fermentation. However, very little information is available on the proteomic profile, phenolic flavonoid compounds and microorganisms during the traditional fermentation of sugarcane wine (Loung). No reports are available on biomolecules present in traditional alcoholic drinks

of sugarcane produced in Madingou.

2. Methods

2.1. Raw Materials and Production of Traditional Loung

The samples of fermented wine were collected in Madingou, in the Department of Bouenza, in the south of the Republic of Congo. The traditional production of sugarcane wine took place in four main stages including harvesting, preparing the sugarcane, extracting and fermenting the juice. Briefly and as reported by traditional producers, after harvesting the sugar cane, the sugarcanes are peeled and then cut into pieces. The pieces of sugarcane are then crushed or ground using a wooden press whose lever serves as a press. The sugarcane juice is collected in a container fitted with a funnel, in which plant leaves are placed which filter it to remove the sugarcane debris. The juice collected is then combined with the previously peeled stems of *Costus after* and crushed corn to speed up the fermentation process. The mixture is kept under the conditions of fermentation. The wine is then considered ready to consume after 6 to 8 days.

2.2. Determination of pH, Acidity Titratable and the Distillation of Sugarcane Wine

The fermentation was monitored and the pH of the ginger wine was determined by direct measurement using a pH meter (HANNA) (T0, T1, T2, T3, T4, T5, T6, T7, T8, T9 and T10). The titratable acidity of the ginger wine, expressed as tartaric acid content per unit volume, is determined during fermentation by titrimetry using a 0.05N sodium hydroxide solution, in the presence of 1% phenolphthalein as an indicator. The calculation of the degree of alcohol has been done at the end of fermentation. Using a 500 mL test tube, 250 mL fermented ginger wine was measured, poured into a 1 L flask, and then connected to the refrigerant. The fermented drink was heated to the boil. Distillation is stopped after collecting more than 3/4 of the volume of the test tube. For this, the heating is stopped, the flask is cooled to remove the remaining wine and the distillate is then brought to room temperature of the wine. The percentage of alcohol is evaluated at 20˚C by using an alcoholometer.

2.3. Isolation of Microorganisms

The Loung freshly sampled were transported in a cooler and sent to the laboratory as quickly as possible. Dilutions were done, and microorganism suspension was streaked with Mossel medium supplemented with polymyxin B. Enumeration of colonies was done in triplicate on Plate Count Agar (PCA). The Petri dishes were incubated at 37˚C for 24 h to 48 h. After the first isolation on Petri dishes, each colony of a different appearance was separately isolated. Purification of the isolates was rigorously done by successive and alternating subcultures. Purity was estimated by using a microscope for morphological characterization. Gram status was determined by using 3% KOH. Sporulation, hydrogen peroxide

(H_2O_2), and oxidase tests were used for biochemical characterization.

2.4. Determination of Enzymatic Activity and Identification by Using MALDI-TOF

Before detecting exogenous proteins, some enzymatic activities (proteolytic, amylolytic and cellulolytic activities) have been done. Some *Bacillus* isolates were assessed for the ability to secrete proteases as described and modified by Kaya-Ongoto *et al.*, 2019 [12]. Briefly, 1 g of agarose was weighed and mixed with 100 mL of PBS. The mixture was heated in a microwave for 3 min until agarose was completely dissolved and then cooled in a water bath at 40˚C. Then 10 mL of skim milk was added to the mixture. After homogenization, the mixture was poured into the Petri dishes. Once solidified, wells were carefully and aseptically generated into the gels. A volume of 50 µL of the overnight culture supernatant is deposited in the wells made on the medium composed of 1% agarose gel, 0.01 M PBS, pH 7.4 and supplemented with skimmed milk. The Petri dishes are incubated at 37˚C for 24 hours. The presence of proteolytic activity is detected by a clear halo around colonies indicating hydrolysis of casein. The halo diameter was measured.

To assess the amlylolytic, and pectinolytic activity of isolates, an overnight culture in LB medium was carried out on Petri dishes. Then a young bacterial colony was deposited on the surface of a solid LB medium separately supplemented with 1% of starch 0.5% of pectin. The Petri dishes were incubated 24 h to 48 h. The revelation is made with Lugol. A test is positive if there is a clear zone around the colony corresponding to a lysis range. The percentage of enzymatic activity was determined according to the following formula: % = DT-DC/DT with DT: Total lysis diameter (Lysis area + Colony diameter); DC: Colony diameter; %: Percentage of lysis.

In order to identify protein secreted in sugarcane wine (Loung), a volume of 500 mL was centrifuged three times at 15,000×g for 15 min at 4˚C, filtered through a 0.2-µm pore size polytetrafluoroethylene (PTFE) membrane (Millipore) and then concentrated by using Millipore concentrator with cutoff threshold at 5 kDa. Very quickly after obtaining the concentrate, 100 µL of Loungouila supernatant was spread in Petri dishes to control the presence of some residual bacterium or not. Colorimetric protein determination with coomassie blue (the Bradford Assay, BioRad) has been used to quantify the amount of protein in samples. The final volume (2.5 to 5 mL) has been assessed for MALDI-TOF techniques. Sequences have been obtained and analyzed by using the NCBI platform (https://www.ncbi.nlm.nih.gov/) and by performing BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

2.5. Determination of Total Polyphenols and Flavonoids

The reagent used is the "Folin-Ciocalteu" reagent. The total polyphenolic compounds are determined as follows. 0.1 mL of the plant extract is introduced into

the Eppendorf tube, the extract is then diluted with 0.9 mL of distilled water then 0.9 mL of the Folin-Ciocalteu reagent (1N) is added then immediately 0.2 mL of a Na_2CO_3 solution (20%) is added. The mixture obtained is incubated at room temperature for about 40 minutes, protected from light. The absorbance is then measured using a spectrophotometer at 725 nm with a methanol solution used as a blank. Calibration line was previously carried out before analysis with gallic acid under the same conditions as the samples to be analyzed. The results obtained are expressed in mg gallic acid equivalent per gram of dry matter (E AG/g Ms).

In terms of the flavonoids the reagents used consist of the colorless solution of sodium nitrite ($NaNO_2$, 5%) and aluminum chloride ($AlCl_3$, 10%). The total flavonoids are evaluated by colorimetry, 250 µL of the extract and 1 mL of distilled water are successively introduced into a 10 mL flask. At the initial time (0 minutes), 75 µL of a solution of $NaNO_2$ (5%), and after 5 minutes 75 µL of $AlCl_3$ (10%) are added. After 6 minutes, 500 µL of NaOH (1N) is added and 2.5 mL of distilled water is added successively to the mixture. A calibration curve is drawn up with standard solutions of catechin prepared at different concentrations. The absorbance of the mixture obtained is directly measured with a UV-visible spectrophotometer at 510 nm and the results are expressed in mg equivalent catechin / gr of dry matter (EC/g Ms).

2.6. The Evaluation of Emulsion Index (E24), Biosurfactant Determination and Antagonistic Effects

The production of biosurfactants is associated with the emulsification of hydrocarbons. The emulsion index (E24) was calculated as an indicator for Biosurfactants production. McFarland standards were used as a reference to adjust the turbidity of bacterial culture. The E24 was investigated by adding gasoline, diesel fuel with directly with Loung sample or Bacterial LB medium in 1:1 ratio (v/v). The solution was vortexed for 5 min and incubated for 24 h. The emulsification percentage was calculated through the height of emulsion layer. All the experiments were performed in triplicates. E24 = Height of emulsion layer/ Total height of solution x100. In terms of biosurfactant antagonistic effect against pathogens, the LB medium was prepared and poured on Petri dishes. After the solidification of the medium, a volume of 0.1 ml of the bacterial suspension is seeded throughout the entire box. After drying, 20 µL of biosurfactant extract was deposited in three (3) different locations on a box. The dishes were incubated at 37˚C. for 24 h and the diameters of the inhibition halos were measured.

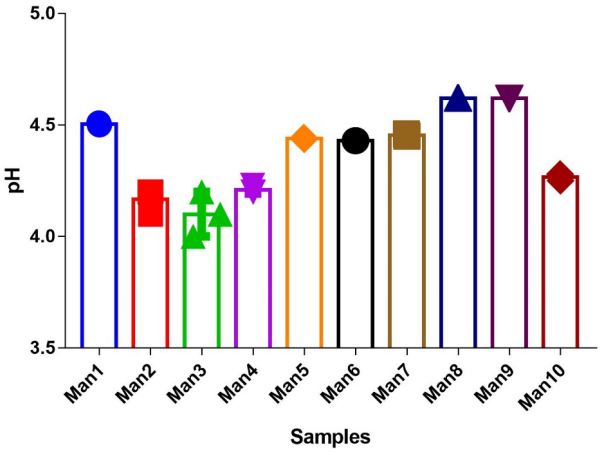
3. Results

3.1. Biochemical Features

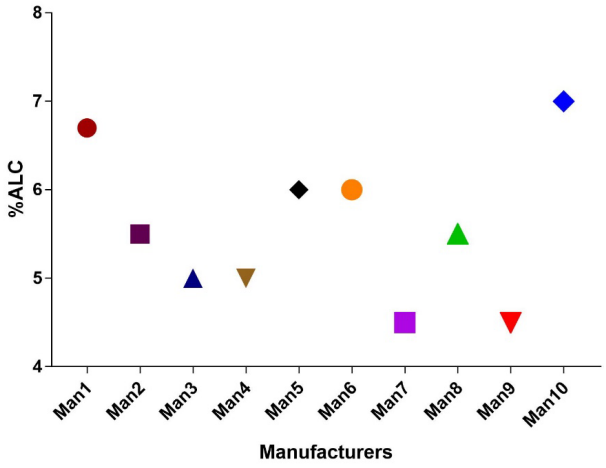
In order to find out the acidity of "Loung", we tested and analyzed 10 samples collected from 10 manufacturers with more than 10 years of experience. The pH values were taken on the seventh day of fermentation. The pH values have been

grouped together in the figure above showing that all the pHs of the drinks are between 4.2 ± 0.1 and 4.7 ± 0.1 representing an acidic pH. The analysis of variance shows that there is a significant difference between the pH of the drinks of the 10 manufacturers by the respondents (Figure 1(a)). The alcohol content was determined by the distillation method using a hydrometer. The figure below shows us the values obtained which are between 4.5% and 7%. (Figure 1(b)).

Three out of ten manufacturers were followed to study the variation in pH during fermentation and the total acidity (Figure 2). These drinks were subjected to the pH assay at the rate of 3 measurements per day and per sample (Figure 2(a)) and three dosages per sample for total acidity (Figure 2(b)); it emerges that the initial pH of 6.5 on the first day decreases as the fermentation continues and stabilizes around 4.25 ± 0.10 on the seventh day and drops to 3.5 ± 0.05 on the 9th day (Figure 2(a)). In terms of the acidity the values are between 1.02 g/L and 6.421 g/L (Figure 2(b))

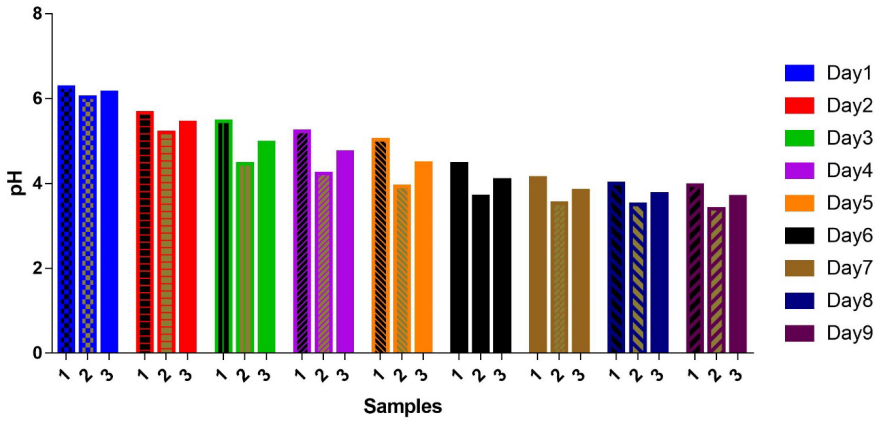


(a)

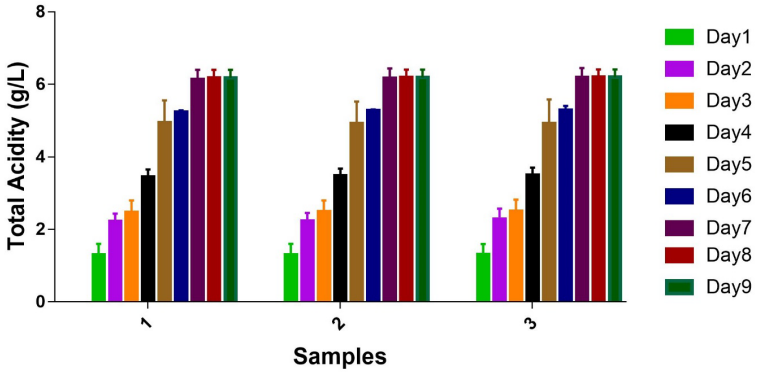


(b)

Figure 1. Evaluation of pH (a) and Alcohol Content (b). Man1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 are manufacturers that were selected for sampling.



(a)



(a)

Figure 2. Monitoring of pH and titratable acidity during the fermentation of sugarcane juice. 1, 2 and 3: manufacturers. Colors are mentioned the day of sampling.

As mentioned in methods the total polyphenol compound and flavonoids have been carried out. As a result, we showed that the polyphenol concentration of Loung varies from 14.9 to 20.5 mg Eq AG/g Ms after 10 days of fermentation (**Figure 3**). The flavonoid concentration varies from 1.70 to 5.22 mg Eq Cat/g Ms after 10 days of fermentation (**Figure 3**).

3.2. Isolation of *Bacillus* Isolates

46 isolates from Loung have been randomly chosen for future experiments. Macroscopic and microscopic analysis, Gram-positive bacteria with 3% KOH, spore-forming bacteria test, and the positive catalase reaction allowed the classification of working bacterium as candidates from the genus Bacillus. To easily discriminate *Bacillus* spp. At the general level, pure cultured isolates were assessed for their ability to produce extracellular cellulose, amylases, and protease as well. A screening of the cellulolytic amylolytic and proteolytic activities was carried out among 46 isolates with *Bacillus*-oriented phenotype. The presence of enzymatic activities was detected by using cellulose, starch, and casein as the following substrates (**Figure 4**). Among 46 isolates 55% (25/45) were able to show a very interesting clear halo in terms of cellulolytic activity with the per-

centage ranging from 37.3% to 83.9% (Figure 4(a)), and 41.3% amylolytic activity for the percentage ranging from 52.02% to 75%. The degradation of casein by caseinase-type proteases was noted by the appearance of a clear halo around the well in which the supernatant of each isolate to be tested was deposited (Figure 4(c)). The absence of a clear halo around the indicated area corresponds to a negative test (absence of caseinases). More than half of the strains tested produce caseinases. The diameters of each halo were measured and shown in Figure 4(c). 65.21% (30/46) of the isolates tested were found to be positive by the caseinolytic test with zones of inhibition ranging in diameter from 1.10 to 3.25 cm. The May 27 strain produced a large zone of inhibition, 3.25 cm in diameter (Figure 4(d)).

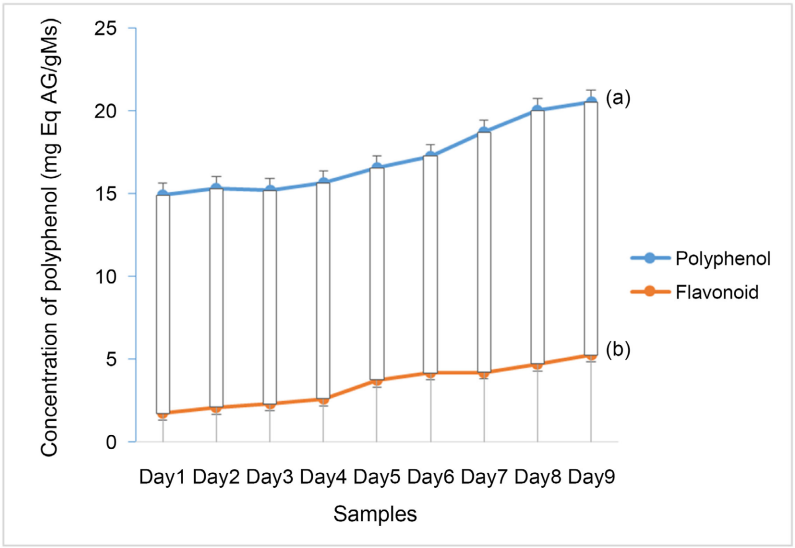
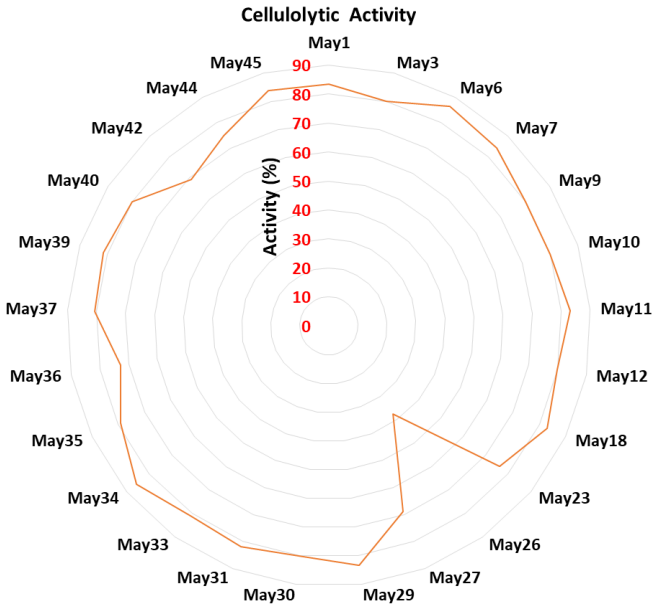
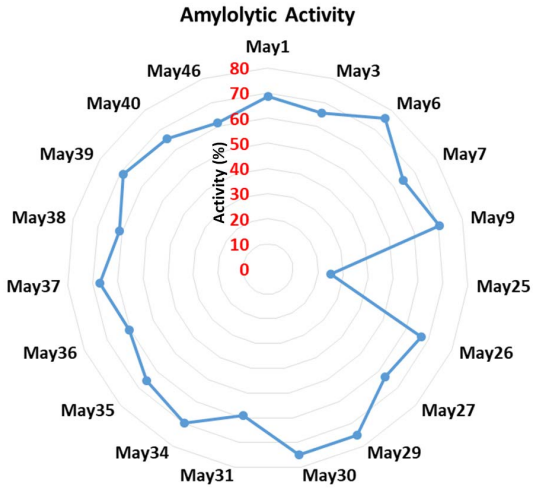


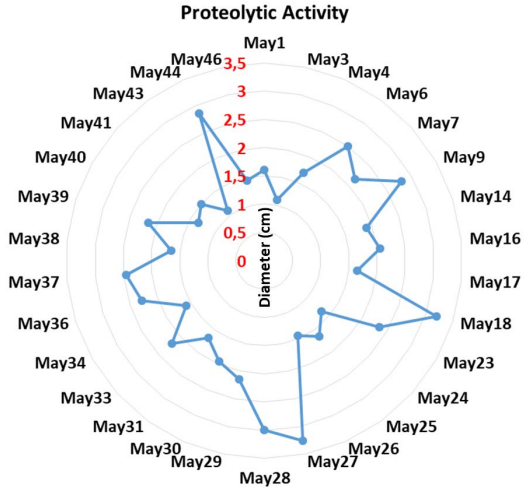
Figure 3. Bioincrease of polyphenol (a) and flavonoids (b) during the fermentation of Sugarcane (Loungouila).



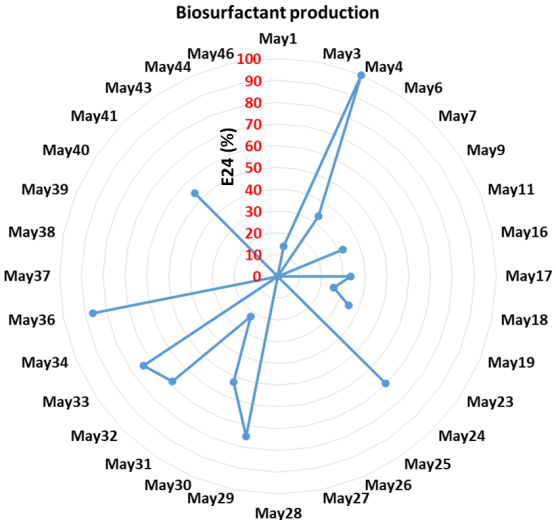
(a)



(b)



(c)



(d)

Figure 4. Cellulolytic activity (a), Amylolytic activity (b), Proteolytic activity (c) and Biosurfactant production (c). May1, … 46: *Bacillus* isolates.

Some isolates have been tested for the ability to produce biosurfactant-like molecules in the extracellular area (**Figure 4(c)**). As results 34.78% (16/46) were able to produce biosurfactants with percentages ranging from 14% to 100% (**Figure 4(c)**).

3.3. Sequence Analysis in Malditof

Proteomic profiling of Loung has been investigated. The concentrate of the sample has been evaluated. The total value was 2.56 g/L. **Table 1** shows the sequences, the percentage homology and the associated strain found in NCBI. Based on sequences, proteins including AprE, SubC, amyE, NprE, CelA and lytF have been identified. The potential Bacillus species that are capable of harboring and to secrete the protein in the extracellular area are mentioned in **Table 1**.

4. Discussion

The fermenting sugar cane at room temperature for a period of seven days, led to a decrease of the pH value to ≤4.2 and an increase in titratable acidity and alcohol content of at least 6.421 g/L (w/v) and 7% respectively. The quality attributes of the alcoholic beverages indicated that pH levels were in the range of 4.15 - 4.25. pH is an important quality parameter in the valorization of traditional fermented beverages. It imparts organoleptic features including color, taste and aroma [13] [14] [15]. The redox potential and microbial growth can be affected in terms of quality control [16]. The levels of pH and titratable acidity observed in the post-fermentation of Loungouila (Loung) alcoholic beverages were comparable to those reported in other studies related to traditional fermented beverages [2] [17] [18] [19] [20].

The polyphenol concentration of Loung increased from 14.9 to 20.5 mg Eq AG/g Ms after 10 days of fermentation. The flavonoid concentration varies from 1.70 to 5.22 mg Eq Cat/g Ms. The molecule of Loung post-fermentation could be efficiently applied to food industrial production, and fermentation significantly increased the antioxidant activity and flavonoid content of Loung. Many traditional fermented foods and beverages have been shown to harbor at the end of fermentation antioxidant activity and flavonoid. This includes ginkgo seeds [21] and Ginger Juice [11]. The big increase in flavonoid and polyphenol have been attributed to the enzymes secreted by *Bacillus* and Saccharomyces species [11]. In this current study, 46 isolated associated with Bacillus sp have been isolated. Short fragment sequences from MALDI-TOF Technique Loung supernatent, have allowed to randomly identify *Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, *B. sonorensis*, *Bacillus glycinifermentans*, *B. pumilus*, *B. holotolerans*, *B. tequilensis*, *B. pumilus*, *B. mojavensis*, *B. cellulosilyticus*, *Paenibacillus anaericanus*, *P. segetis*, *B. mesophilus*, *B. siamensis*, *B. mojavensis*, *B. velezensis*, *P. humicus*, *P. borealis*, *P. tritici*, *P. typhae*, *B. cereus*, *B. toyonensis*, *B. mycoides*, *B. wiedmannii*, *B. thuringiensis.* Some isolates were able to produce Biosurfactant like molecules as previously demonstrated with the genus *Bacillus* [22] [23]

Table 1. Protein profiling of Loungouila by using MALDI-TOF technique.

Sequences	Proteins and functions (NCBI, Uniprot)	Identity % (NCBI)	Signal peptide (Sec/SPI) (SignalP5.0)	Potential Strains
- VPYGVSQIKA - YPGKYPS VIAVG	AprE Peptidase S8	100%	96.37%	*Bacillus amyloliquefaciens* *Bacillus subtilis*
- DSVIAV - STALKQAVDK	SubC Peptidase S8	100%	97.59	*Bacillus licheniformis* *Bacillus sonorensis* *Bacillus subtilis* *Bacillus glycinifermentans*
- IEWAISN - YTGSNVKVAVID	AprE Peptidase S8	100%	97.2%	*Bacillus pumilus* *Bacillus holotolerans* *Bacillus subtilis* *Bacillus tequilensis*
- SPINQV - APHVFLENYK	AmyE Alpha-amylase	100%	96.66%	*Bacillus subtilis*
- AVDAHY - AKAALIQSAR - NQPDNY	NprE Neutral protease	100%	92.16%	*Bacillus subtils* *Bacillus pumilus* *Bacillus mojavensis* *Bacillus subtilis* *Bacillus amyloliquefaciens*
- ATR - AGR - DDWGSNMIR - VEVLSR - PSDGWATAPR - VIVDWHVHAPGDPR	CelA Endoglucanase A	100%	97%	*Bacillus cellulosilyticus* *Paenibacillus anaericanus* *Paenibacillus segetis* *Bacillus mesophilus*
- FNVTAQQIR - VTSVSR - AQLGVPYR	LytF Peptidoglycan endopeptidase	100%	*93.37%*	*Bacillus subtils* *Bacillus siamensis* *Bacillus mojavensis* *Bacillus velezensis*
- LNPASEWGTR - TAPTFDQSLIASEASGQK	Mut Putative Mutanase	100%	*98.01*	*Paenibacillus humicus* *Paenibacillus borealis* *Paenibacillus tritici* *Paenibacillus typhae*
- DSKAFIDVSAATLWTAPDSLR - NDGTFYR - VLVHGQPTPR	Gamma-D-glutamyl-L-lysine dipeptidyl-peptidase YkfC	100	*99.26%*	*Bacillus cereus* *Bacillus toyonensis* *Bacillus mycoides* *Bacillus wiedmannii* *Bacillus thuringiensis*

[24] [25] [26]. The presence of biosurfactant confers a safe wine of Loung since some antagonistic effects of biosurfactant against *Shigella flexneri*, *Salmonella* sp, *Staphyloccoccus aureus* and *Pseudomonas* sp. have been tested (data not

showed). The biochemical structure of the purified surfactin-like molecules is keeping on the way of characterizing by electrospray ionization mass spectrometry (ESI-MS) and high-resolution ESI Q-Tof mass spectrometry (Q-Tof MS)

Molecular profiling of Loung has been allowed to identify some proteins secreted by generally recognized as safe (GRAS) bacteria. The identified proteins include AprE, AmyE, CelA, LytF, YkfC and NprE. The genes encoding neutral (*nprE*), and alkaline (*aprE*) protease are two major extracellular proteases in *Bacillus* belonging to group I [27] whose expression is directly regulated by several pleiotropic transcriptional factors [28]. This work allowed to predict that AprE (Subtilisin, an extracellular alkaline serine protease), AmyE (Alpha-amylase AmyE) and NprE (Extracellular zinc metalloprotease) could have a network interaction even in extracellular area in string prediction (https://string-db.org/) [29]. The Peptidoglycan endopeptidase LytF; Cell wall hydrolase that cleaves gamma-D-glutamate-meso-diaminopimelate that can bind in peptidoglycan and a CelA from *Bacillus* species which is cellulose-binding endo-beta-1,4-glucanase have been identified [30] [31]. In cells growing in a rich, complex medium, the aprE and nprE genes are strongly expressed only during the post-exponential growth phase and were clearly identified using the MaldiTof technique. The post-growth events of the isolates randomly taken demonstrated the presence of AprE, NprE and AmyE in the extracellular medium. Enzymes including amylases, cellulases, hemicellulases, lipases, pectinases, oxygenases, dehydrogenases, lignin-modifying enzymes, and mutants are secreted by bacteria of the genus *Bacillus*, *Paenibacillus* or *Lysinibacillus* in the extracellular medium [11] [32] [33] [34]. The SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP/) allowed to predict the presence of signal peptides of the Sec pathway and the location of their cleavage sites in proteins from Gram-positive bacteria [35]. Their presence of proteins in Loung testifies to the great bacterial activity of *Bacillus* during fermentation with regard to the digestion of protein fibers [36]. In this work we have confirmed by the positive test for milk casein, starch and cellulose, testifying the presence of these protease, amylases and cellulase in Loung.

In addition, it is important to note that the short fragments of sequence obtained after MALDI-TOF of the protein AprE, SubE, NprE, LytF, CelA and AmyE have been associated with 2 or more species of *Bacillus* belonging to *B. subtilis* Group with a conservation percentage of 100%.

The Gamma-D-glutamyl-L-lysine dipeptidyl-peptidase (YkfC) [37] has been found in the total extract of Loung. The protein is highly conserved the *Bacillus* specie belonging to the *B. cereus* group including *B. toyonensis*, *B. mycoides*, *B. wiedmannii*, *B. thuringiensis* [27]. Mut, a putative mutanase (Mut) in *Paenibacillus* group has been found [38].

5. Conclusion

Our findings have demonstrated the scientific contribution in terms of the valorization of the traditional fermented beverage of Loung. It should be noted that

important biomolecules can be isolated in the context of biocatalysis, of health for a highly biological industry. The lesson learned of the different stages of manufacture of traditional beverages, the knowledge of introns, and the identification of bacterial and yeast strains constitute some biochemical approaches that could give added value to fermented food and beverages biotechnology.

Data Availability

The Excel sheets including the data used to support the findings of this study are available from the corresponding author upon request.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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