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The use of earthworm flour for lactic acid biomass production

Liliana Serna Cock[1,2]*, Carlos Andrés Rengifo Guerrero[2] and Miguel Angel Rojas Restrepo[2]

[1]Carrera 32 Via Candelaria, Palmira, Valle del Cauca, Colombia.
[2]School of Engineering and Management, National University of Colombia, Palmira Campus, Colombia.

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The potential use of Californian red earthworm flour as a nitrogen source for the production of lactic biomass was assessed. Three fermentation substrates, earthworm flour (EF), earthworm flour + yeast extract (EF + YE) and a commercial substrate MRS (control) were used. The substrates were formulated using 60 g L^{-1} of glucose as the carbon source and 34 gL^{-1} of earthworm flour as the nitrogen source. *Weissella confusa* was used as lactic acid bacterium. Nine batch fermentations were performed at 32°C and 100 rpm for 4 h, and the kinetics of the biomass concentration, lactic acid concentration and substrate consumption were compared. No differences were observed in the biomass concentration of the EF and EF + YE substrates, and final concentrations of 1.36 and 1.47 g L^{-1} were obtained, respectively. The lactic acid concentration did not differ significantly between EF + YE and the commercial substrate, and values of 4.79 and 4.33 g L^{-1} were obtained, respectively. These results suggest that earthworm flour can be a low-cost alternative for lactic acid biomass production.

Key words: Earthworm flour, *Weissella confusa*, lactic acid bacteria.

INTRODUCTION

Lactic acid bacteria (LAB) are a very important group of microorganisms that have been used for centuries in the production of fermented food; these bacteria have been used in preservation processes, as they can prevent the growth of pathogenic microorganisms (Divya et al., 2002; Gillor et al., 2008). In fermented food products, LAB are responsible for acidification due to the production of acids such as lactic and acetic acids, and the production of bio-protector compounds such as bacteriocins and hydrogen peroxide (Gulahmadov et al., 2006). Studies have highlighted the benefits of using LAB in probiotic products, for example, LAB produce bacteriocins that prevent the growth of the microorganisms responsible for disease and food decay (Serna et al., 2010; Zhu et al., 2009; Divya et al., 2012). LAB are isolated from sources rich in nutrients such as plants, animals, fermented foods, human and animal gastrointestinal tracts and the female genital tract (Zhu et al., 2009). These microorganisms are considered to be nutritionally demanding and require complex substrates for their growth (Lee et al., 2011; Savijoki et al., 2006). In the fermentation industry, the substrates for growth usually constitute the majority of the costs of microbial biomass production and its bioproducts (Kurbanoglu and Algur, 2002). In the biotechnological production of LAB, different substrates have been used such as glucose, lactose and starch (Sheng and Xia, 2006). However, these substrates are not economically viable, not only because the pure substrates are expensive and require the addition of complex nitrogen sources but also because natural polysaccharides require physi-

*Corresponding author. E-mail: lserna@unal.edu.co.

Abbreviations: EF, Earthworm flour; **EF + YF,** earthworm flour + yeast extract.

cochemical or enzymatic pre-treatment before they can be fermented (Young- Jung et al., 2004).

In the LAB and lactic acid production process, 68% of total production costs are associated with the cost of the raw material, and 38% of those costs correspondto yeast extract (Djukic - Vukovic et al., 2012). Therefore, nitrogen sources tend to be the most expensive constituent of the culture media.

Vasquez et al. (2004) indicate that the problem associated with lactic acid bacteria and their metabolites production on industrial scale, is due to the high demand for nutritional sources such as commercial culture medium (MRS, TGE, APT). The culture medium containing peptones, bactopeptonas and meat extract are expensive. The search for alternative substrates that would allow for the optimum LAB growth while maintaining favourable production costs has been the subject of study of several authors (Qi and Yao, 2007; Serna et al., 2007; Guerra et al., 2001). Guerra et al. (2001) evaluated the ability of *Lactococcus lactis* and *Pediococcus acidilactici* strains to produce bacteriocins in diluted and concentrated serum by evaluating the effect of the total content of sugars, nitrogen and phosphorous. Anthony et al. (2009) evaluated the influence of the substrate composition and the culture conditions on the production of LAB and its bacteriocins using a *Bacillus licheniformis* AnBa9 strain. For producing bacteria, Taskin and and Kurbanoglu (2011) evaluated chicken feathers hydrolysates as nitrogen source of low cost, and Kurbanoglu and Canli (2011) assessed as nitrogen source, sheep horns hydrolysates for economic production of glucose oxidase.

The Californian red earthworm (*Eisenia foetida*) flour could become an economically feasible alternative nitrogen source in fermentative processes such as lactic acid biomass production because it has high contents of nitrogen and proteins and a high reproduction rate (Vielma et al., 2007). Vermiculture is an activity that uses the Californian red earthworm as an alternative for recycling organic waste from different sources and as a nonconventional source of proteins and other nutrients at low cost (Vielma et al., 2003). The practice of vermiculture has a century old, and it has been used with environmental objectives such as waste management, soil detoxification and regeneration, and sustainable agriculture (Sinha et al., 2002).

Accordingly, the objective of this work was to evaluate the potential use of Californian red earthworm flour as a nitrogen source for the low-cost production of lactic acid biomass. Its potential use was measured in terms of its nutritional composition and kinetic parameters such as the specific growth rate (μ), biomass concentration, biomass yield ($Y_{x/s}$) and substrate consumption. In addition, concentration and lactic acid yield ($Y_{p/s}$) was measured.

The values of the mentioned variables were compared with the kinetic parameters obtained from a commercial substrate.

METHODOLOGY

Earthworm flour extraction

The Californian red earthworm (*E. foetida)* was obtained from Rancho J, a commercial vermiculture facility located in Buga, Cauca Valley, Colombia. 2 kg of adult worms was obtained from a composting pile composed of cattle and pig excreta. The earthworms were treated and sacrificed according to the methodology proposed by García et al. (2009) and Sogbesan and Ugwumba (2008). The earthworms were washed twice with tap water and left for 24 h in a container with distilled water and constant oxygen bubbling to remove the waste in their digestive tracts. They were sacrificed through a thermal treatment at -20°C (Electrocool LG, Mexico - Ciudad de Mexico) for 18 h. Afterwards, they were dried in a convection oven (Binder ED115, Germany) at 60°C for 24 h. The sample was then removed and milled (Fritsch Germany, particle size of 1 mm, 8000 rpm) to obtain the earthworm flour (EF).

Determination of the chemical composition

The earthworm flour contained dry matter, ash (AOAC, 1990, 942.05), minerals, organic carbon (Walkley and Black method, 1934), phosphorous and boron (Dawson, 1986), (UV-Vis spectroscopy), potassium, calcium, magnesium, sodium, other minor elements such as copper, iron, zinc and magnesium (Varga and Kolodziej, 1974), (atomic absorption spectroscopy), and crude protein (Kjeldahl, 1883) and ether extract (Soxhlet, AOAC, 1990, 920.39).

Lactic acid bacterium

A cryopreserved strain of *Weissella confusa* obtained in studies by Serna et al. (2010) was used. The strain was attached to each substrate after three successive generations. 24 h cultures, incubation temperature of 32 ± 0.5°C and 10% of inoculum, with respect to working volume were used.

Fermentation substrates

Three fermentation substrates were formulated: a control substrate (the MRS commercial substrate, Merck, Germany), the EF substrate (34 g L^{-1}) and EF + YE substrate [34 g L^{-1} of earthworm flour + 4 g L^{-1} of yeast extract (Oxoid, UK)]. All of the substrates were supplemented with 60 g L^{-1} of glucose as a carbon source. Nine batch fermentations were performed (three for each fermentation substrate). Once the substrates were prepared, they were placed in 1000-ml Erlenmeyer flasks with a working volume of 200 ml and were sterilised for 15 min at 121°C (Essem, 250A). The Erlenmeyer flasks remained under elliptical agitation at 100 rpm for 4 h at 32 ± 0.5°C (VWR Incubating Orbital Shaker VWR model 5000I, USA). During the course of the fermentations, the pH was adjusted to pH 6.0 using 1 M NaOH, according to the methodology described by Serna et al. (2010).

Kinetics of the substrate consumption, biomass formation and lactic acid concentration

For each substrate, 10-ml samples were aseptically taken at 0, 1, 2, 3 and 4 h of fermentation (time 0 corresponding to the time the inoculum was added). The samples were centrifuged at 5000 rpm for 10 min (Eppendorf Centrifuge - 5804R, Germany). The supernatant was used to measure the lactic acid concentration and

Table 1. Chemical and mineral composition of the earthworm flour (*Eisenia foetida*).

Parameter	Value
Dry matter (%)	93.7
Protein (%)	55.6
Nitrogen (%)	8.91
Ether extract (%)	8.09
Ashes (%)	8.69
P (%)	2.40
K (%)	1.58
Ca (%)	1.05
Mg (%)	0.81
Na (%)	0.73
Cu (ppm)	0.26
Fe (ppm)	5.07
Zn (ppm)	1.38
Mn (ppm)	0.22
B (ppm)	13.9

substrate consumption kinetics. The lactic acid was measured by reflectometry (Reflectoquant RQFlex plus 10 Merck, Germany) using reaction test strips. The substrate consumption was determined by spectrophotometric measurements (Genesys 10UV, EEUU) of the reducing sugars using the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959). The precipitate was washed two times with a 0.9% NaCl solution and used to determine the biomass by dry weight (AOAC, 1990, 923.03). With the data obtained for the biomass concentration, lactic acid concentration and reducing sugars, the following kinetic parameters were calculated: the specific growth rate (μ) which is calculated from the biomass formation kinetic curve, and the biomass yield ($Y_{x/s}$) and the product yield ($Y_{p/s}$), which are calculated using Equations 1 and 2, respectively. The percentage of reducing sugars consumed (RSC) was calculated using Equation 3:

$$Y_{x/s} = \frac{X - X_o}{S_o - S} gg^{-1} \quad (1)$$

$$Y_{p/s} = \frac{P}{S_o - S} gg^{-1} \quad (2)$$

$$RSC = \frac{(S_o - S) * 100}{S_o} \ \% \quad (3)$$

Where X_0, is the initial biomass concentration (g L^{-1}); X, is the final biomass concentration (g L^{-1}); S_0, is the initial reducing sugars concentration (g L^{-1}); S, is the final reducing sugars concentration (g L^{-1}); and P, is the maximum concentration of lactic acid (g L^{-1}). The final concentrations were determined when P was maximal.

Statistical analysis

A unifactorial model with three replicates was used, where the type of substrate was a factor with three levels (control, EF and EF + YE). The response variables were the biomass concentration, the lactic acid concentration and the substrate consumption, which were measured at 0, 1, 2, 3 and 4 h of fermentation. In addition, the kinetic parameters specific growth rate (μ), product yield ($Y_{p/s}$) and biomass yield ($Y_{x/s}$) were calculated. The results were analysed with the statistical package SAS 9.2 for Windows (SAS Institute Inc.(1993), Cary, NC, USA). The average values were compared with the Tukey test, with a probability of $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of earthworm flour

The results for the percentages of dry matter, nitrogen, protein, ashes, ether extract and earthworm flour minerals are shown in Table 1. The earthworm flour had high protein content (55.6% dry matter). Similar results were reported in previous studies, where the importance of the nitrogen and protein content of earthworm flour was highlighted (Vielma et al., 2007; Sogbesan and Wgwumba, 2008; Garcia et al., 2009). Horn et al. (2007) indicated that the nitrogen content is the most important parameter in the culture media. Likewise, Haq et al. (2008) mentioned that nitrogen is an important quantitatively bioelement, which is required by many microorganisms during fermentation for protein synthesis. Cellular growth is directly influenced by the composition of the fermentation substrate.

According to Vielma et al. (2007), earthworm flour has a good content of essential amino acids, vitamins and minerals that are necessary for optimal cellular growth of lactic acid bacteria. The ash content in earthworm flour was high, and similar to ash content reported by Vielma et al. (2007). Authors such as Horn et al. (2007) and Djukic - Vukovic et al. (2012) used cod viscera and liquid vinasse in lactic acid fermentation, and they found that the ash content was 10.1 and 14.4%, respectively. According to Reddy et al. (2011), high ash contents in the sample could be an advantage because it provides mineral elements that are necessary for the growth of microorganisms. The mineral composition of earthworm flour showed contents of phosphorous, potassium, calcium, zinc and boron. It is significant that the earthworm flour provides elements such as phos-phorous because it favors the synthesis of phospholipids, nucleic acids and proteins (Taskin et al., 2011).

In addition, calcium and magnesium can have a protective effect on the cell, favoring its viability (Xue et al., 2008); however, authors such as Chotineeranat et al. (2010) claim that the presence of high concentrations of calcium in the fermentation substrate has an adverse effect on the cell, lowering its growth and biomass yield rate. Furthermore, elements such as Mg, Ca, Mn, Fe and Zn, stimulate the lactic acid production and the growth of lactic acid bacteria (Djukic - Vucokic et al., 2012). Kurbanoglu et al. (2001) report that element such as iron has a toxic effect on microorganisms when used in high

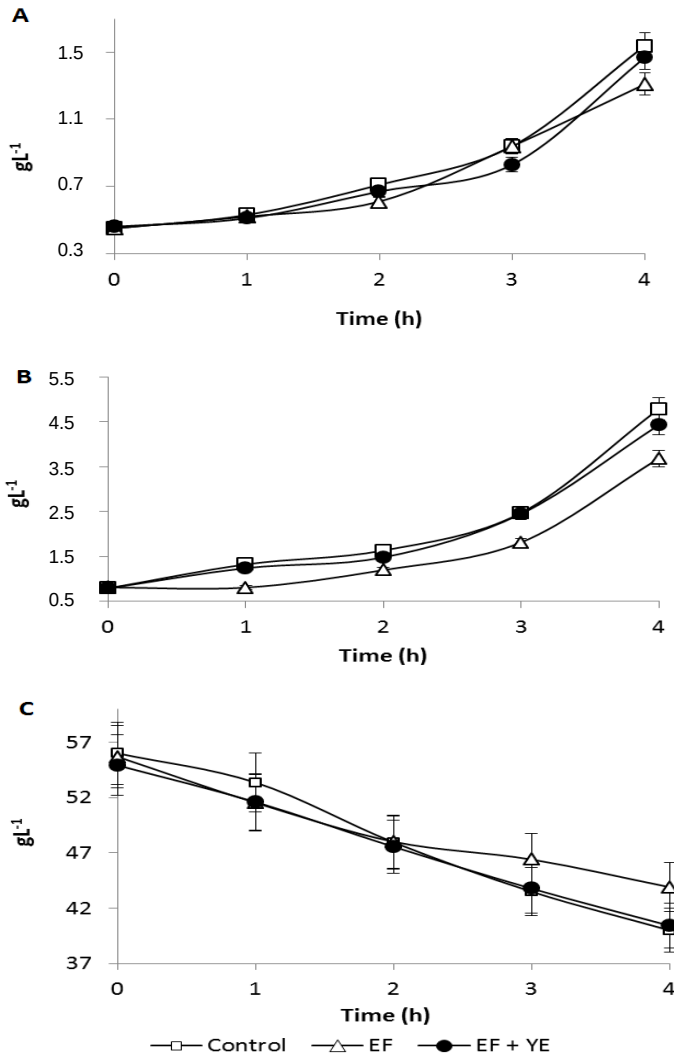

Figure 1. a) Kinetics of the biomass concentration; **b)** kinetics of the lactic acid concentration; **c)** kinetics of the *Weissella confusa* substrate consumption for the three evaluated substrates.

concentrations, inhibiting the cellular growth. According to Djukic - Vukovic et al. (2012), the optimal concentration of Fe and Ca for growth of LAB is ≤ 4 and ≤ 8000 mg L^{-1}, respectively. When using 34 gL^{-1} of earthworm flour, the contribution of Fe and Ca is 0.17 and 357 mgL^{-1}, respectively; this is an optimal range for growth of LAB. Van Niel and Hahn - Hagenrdal (1999) showed in a strain of *L. lactis*, that when the culture medium is supplemented with 2 to 4 ppm of Fe, biomass and product yields are improved.

Formulation of substrate and kinetic parameters

The earthworm flour did not show good solubility during the preparation of the fermentation substrate. When preparing the fermentation broth with earthworm flour as the nitrogen source, the formation of precipitates occurred after sterilization. Vielma and Medina (2006) claim that the earthworm flour contains proteins with different percentage (%) of solubility that depend on the pH of the medium. Garcia et al. (2010) suggest that when the peptides that constitute the culture medium are smaller, the precipitation and turbidity effects caused by the sterilization will be lower. These authors also claim that the precipitation effect could be caused by the combination of calcium and phosphate ions present in the peptones. Biomass values were corrected by subtracting the fraction of earthworm flour precipitated during centrifugation.

The fraction of earthworm flour was calculated determining its weight in trials where no inoculum was used. Figure 1 shows the kinetics of the biomass concentration, lactic acid concentration and substrate consumption. Table 2 shows the specific growth rate (μ), biomass yield ($Y_{x/s}$), product yield ($Y_{p/s}$) and the percentage of reducing sugars consumed (RSC). Table 3 shows the analysis of the averages obtained by the Tukey test. The substrate consumption did not exhibit significant differences between the three treatments ($p > 0.05$) at any of the fermentation times. The highest consumption value corresponded to the control substrate, followed by the EF + YE substrate (Table 3). The highest biomass concentration (Figure 1a) was obtained at the fourth hour of fermentation using the control substrate (1.54 g L^{-1}), followed by EF + YE (1.47 g L^{-1}) and EF (1.31 g L^{-1}). The statistical analysis did not show statistically significant differences between the three types of substrates ($p>0.05$). The lactic acid concentration (Figure 1b) did not show significant differences between the control substrate and the EF + YE substrate. The highest lactic acid concentration was obtained with the control substrate (4.79 g L^{-1}), followed by EF + YE (4.33 g L^{-1}) and EF (3.68 g L^{-1}), at the fourth hour of fermentation.

As shown in the Figure 1a and Table 2, the biomass concentration, biomass yield ($Y_{x/s}$) and the product yield ($Y_{p/s}$) were slightly higher with the commercial substrate ($p>0.05$), followed by EF + YE and EF (Table 2). This growth assumes that when using earthworm flour as the fermentation substrate, the required nutrients are provided, such as nitrogen, vitamins and some amino acids that are necessary for bacterial growth (Altaf et al., 2005). In general, all of these elements are supplied as complex constituents such as the yeast extract. However, the results show that using earthworm flour as the source of nitrogen and minerals, high biomass yield and biomass concentration are obtained, even without the use of yeast extract. Serna et al. (2010) found similar biomass and product yields using a *W. confusa* strain (0.062 and 0.46 g g^{-1}, respectively) and a commercial substrate. However, the same authors found higher yields when using milk supplemented with yeast extract, with yields of 5.98 g g^{-1} for the biomass and 0.73 g g^{-1} for the product. Mondragon-Parada et al. (2006) found similar yields

Table 2. Specific growth rate (μ), biomass yield ($Y_{x/s}$) lactic acid yield ($Y_{p/s}$) and percentage of reducing sugars (RSC) of *Weissella confusa* in control, EF + YE and EF substrates.

Substrate	μ (h^{-1})	$Y_{x/s}$ (gg^{-1})	$Y_{p/s}$ (gg^{-1})	% RSC
Control	0.3034	0.069 ± 0.011	0.301 ± 0.055	28.59
EF + YE	0.2811	0.068 ± 0.007	0.298 ± 0.030	26.41
EF	0.2729	0.065 ± 0.001	0.253 ± 0.006	21.17

Table 3. Tukey test* (average comparison).

Substrate	Substrate consumption	Lactic acid	Biomass
Control	48.360 ± 6.6560[a]	2.279 ± 1.5332[a]	0.830 ± 0.4372[a]
EF + YE	47.022 ± 5.8291[a]	2.065 ± 1.4077[a]	0.793 ± 0.4079[a]
EF	49.241 ± 6.6560[a]	1.656 ± 1.2037[b]	0.756 ± 0.3573[a]

*Averages with the same letter are not significantly different ($p > 0.05$).

biomass using *Lactobacillus casei*, and milk serum as fermentation substrate (0.063 gg^{-1}). Vasquez et al. (2004) reported yields of biomass 0.057 and 0.089 gg^{-1} using hydrolyzed squid viscera for growth of strains of *L. lactis* and *P. acidilacti*, respectively. Djukic - Vukovic et al. (2012) using liquid vinasse reported higher yields of lactic acid (0.90 g g^{-1}).

Authors such as Nancib et al. (2001), Altaf et al. (2005) and Gao et al. (2006) have emphasised the importance of vitamins as a microbial growth factor. Authors such as Berry et al. (1999) evaluated the growth of *Lactobacillus rhamnosus* in a similar medium as the MRS commercial substrate. They highlighted the importance of the presence of amino acids such as cysteine, asparagine and glutamine in the fermentation substrate, because these compounds provide elements such as sulphur and nitrogen, which are necessary in the reproduction stage. Vielma et al. (2007) found in their study that earthworm flour contains vitamins such as biotin and riboflavin; amino acids such as leucine, glutamic acid and aspartic acid; and elements such as magnesium, sodium and potassium, that favor cellular growth and metabolism. Recent studies have focused on the search for agro-industrial wastes and non-conventional raw materials with biotechnological potential for fermentation processes to lower the cost of the raw materials. Due to the nutritional value of earthworm flour, this raw material can be used as a nitrogen source for low-cost lactic biomass production. Horn et al. (2007) reported that a culture medium capable of stimulating the growth of lactic acid bacteria is probably, a good substrate for the growth of other microorganisms, which have similar nutritional requirements or lower compared with requirements of lactic acid bacteria.

In this study, earthworms were obtained from vermiculture activity; a process in which the worms were fed with agricultural wastes, therefore, production costs of the worm was not significant. In energetic terms, the use of these raw materials requires an adaptation of the previous process. For example, to prepare 1 kg of earthworm flour under laboratory conditions, the total required energetic demand was 41.42 kW h^{-1} and was distributed for the following processes: freezing (2.52 kW h^{-1}), drying (38.4 kW h^{-1}) and grinding (0.5 kWh^{-1}). From the aforementioned, the approximate cost of earthworm flour production is 8.96 USD/kg. Therefore, when 34 gL^{-1} of earthworm flour as fermentation substrate is used, the cost of raw materials for the production of lactic biomass is reduced by 60%, compared to the cost of using commercial substrate. However, the cost estimation was based on operations conducted at a laboratory scale; thus, subsequent studies should be aimed at scaling up this process to the industrial level and determining the economic viability of such a process.

Conclusions

The use of earthworm flour as a fermentation substrate considerably reduces production costs and produces acceptable biomass levels for use in the generation of probiotic products. In addition, the biomass can be used in the food industry for the production of bacteriocins that prevent the growth of pathogens and microorganisms that cause food decay. Thus, Californian red earthworm flour has important biotechnological and economic potential in lactic acid biomass production.

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