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DEVELOPMENT OF BACTERIAL CULTURE MEDIUM FROM AVOCADO SEED WASTE

DESARROLLO DE UN MEDIO DE CULTIVO BACTERIANO A PARTIR DEL RESIDUO DE SEMILLA DE AGUACATE

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Abstract

Avocado (*Persea americana* Mill) seeds, a rich source of starch and micronutrients, are a major waste product from the agroindustrial processing of avocados. We designed and developed an experimental culture medium (ECM) from hydrolysed avocado seeds, supplemented with M9 salts (10% v/v). Breaking of starch granules of avocado seeds due to hydrolysis treatments was analysed by morphology and morphometry of granules. We evaluated the ECM functionality by measuring the growth of *E. coli* as affected by (i) the carbon source (reducing sugars concentration), (ii) the nitrogen source, and (iii) mixing and aeration in a stirred tank bioreactor. ECM containing 13.33 and 20 g/L of reducing sugars reached a biomass production of 1.75 and 2.22 gDCW/L, respectively. Interestingly, the biomass yield from ECM was at least 2.5-fold higher than that obtained using Luria-Bertani Broth (LB) medium (0.23 vs 0.09). In addition, the growth rate increased with the agitation velocity (0.44 h^{-1} at 200 rpm; 0.36 h^{-1} at 150 rpm). Our findings suggest that avocado seeds represent a cost-effective material for producing a sustainable culture medium for bacterial growth of *E. coli* and other strains of interest in biotechnological processes.

Keywords: avocado seed waste, *Escherichia coli*, growth medium, acid hydrolysis, bioreactor.

Resumen

Las semillas de aguacate (*Persea americana* Mill), una fuente rica de almidón y micronutrientes, son uno de los principales productos de residuos del procesamiento agroindustrial de los aguacates. Nosotros diseñamos y desarrollamos un medio de cultivo experimental (ECM) a partir del hidrolizado de las semillas de aguacate, adicionado con sales M9 (10% v/v). El rompimiento de los gránulos de almidón de las semillas de aguacate debido al tratamiento de hidrólisis, se analizó por la morfología y morfometría de los gránulos. También evaluamos la funcionalidad del EMC al medir el crecimiento de *E. coli* considerando: (i) la fuente de carbono (concentración de azúcares reductores), (ii) la fuente de nitrógeno, y (iii) el mezclado y aireación en un biorreactor de tanque agitado. Los medios ECM con 13.33 y 20 g/L de azúcares reductores alcanzaron una producción de biomasa de 1.75 y 2.22 gDCW/L, respectivamente. En particular, el rendimiento de biomasa en el ECM fue 2.5 veces mayor al obtenido con el medio Luria-Bertani (LB) (0.23 vs 0.09). Además, la tasa de crecimiento de *E. coli* se incrementó conforme la velocidad de agitación (0.44 h^{-1} a 200 rpm; 0.36 h^{-1} a 150 rpm). Por lo tanto, nuestros resultados sugieren que las semillas de aguacate constituyen un material aprovechable para la producción de un medio de cultivo para el crecimiento de *E. coli*, y otras cepas de interés en procesos biotecnológicos.

Palabras clave: residuos de semillas de aguacate, *Escherichia coli*, medio de cultivo, hidrolisis ácida, biorreactor.

1 Introduction

Avocado (*Persea americana* Mill), is an important tropical crop and is composed mainly of unsaturated fatty acids, fibre, and vitamins B and E (Dabas *et al*., 2011). World avocado production in 2012 was 4,360,018 tons, and Mexico was the world's largest producer at 1,316,104 tons (FAO, 2014). In avocado industrial processing in the agronomic and food industries, the seeds (16% of the total weight of the fruits) are considered unusable wastes (Ramos-

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Jerz, 2007). Annual production of 2,700 tons of avocado seeds has been estimated (Dabas *et al*., 2011) and this waste lacks appropriate utilization. In fact, avocado seed waste contributes to environmental pollution in the form of methane emission, leachate, and atmospheric pollution by incineration (Asim *et al*., 2014). However, avocado seeds do contain components that could be advantageously used, such as, polyphenols (Wang *et al*., 2010), which possess antioxidant activity, and starch, which constitutes up to 60% of the dry weight of an avocado seed (Kahn, 1987).

Therefore, avocado seeds represent a large carbohydrate reservoir, and thus a valuable carbon source for microbial growth. In addition, avocado seeds contain high levels of nitrogen and fatty acids, and low concentrations of saponins, glycosides, sterols and carnitine (Jimenez-Arellanes *et al*., 2013).

Most commercially available culture media, e.g. Luria-Bertani (LB) medium, are expensive. Despite this, LB medium is popular with bacteriologists because it permits fast growth and good growth yields for many species (Sezonov *et al*., 2007). Particularly, LB medium supports *Escherichia coli* growth to an optical density at 600 nm (OD600) of 7 (Sezonov *et al*., 2007). However, the cost of LB medium and other media has motivated the search for new formulations that support microbial growth. The preparation of media from inexpensive bio-products which are able to support and fulfil nutritional requirements for microbial growth is currently receiving notable research attention (Andualem and Gessesse, 2013). Different materials derived from plants, such as groundnut, sorghum extracts, cassava whey, cereals, and local food waste, have been used to create microbiological growth media (Famureawa and David, 2008).

The increased production of avocado seeds in Mexico and other countries requires the development of sustainable technological applications for this waste material. The presence of starch and other micronutrients in avocado seeds makes them an attractive source for the development of a culture medium for microorganisms. In fact, the medium could theoretically be used for microorganisms that cannot use starch directly (due to the lack of enzymes with amylase activity) but can degrade starch hydrolysates, i.e. *Escherichia coli* (Rosales-Colunga *et al*., 2014). Thus, the particular aim of this study was to develop a culture medium from avocado seeds for the growth of *E. coli* K12 MG1655. This development provides an added value to the productive chain of the avocado agroindustry because of the potential reduction of the environmental impact of seeds as an agronomic industry waste.

2 Materials and methods

2.1 Avocado seed powder (ASP) preparation

Avocado seeds were provided by SIOSI Alimentos Inc. (México). Chopped seeds (*Persea americana* Mill cv. Hass) were spread into a tray and placed in an oven at 60°C (Boekel, USA) until dried. Dry materials were subsequently finely ground using a ball mill grinder (RETSCH®) for 20 seconds. The resulting avocado seed powder (ASP) was stored at room temperature.

2.2 Hydrolysis treatments of avocado seed powder

ASP (100 g/L) was initially treated with hydrochloric acid (HCl) (1.0%, v/v) (Karal, México), without thermal pressure treatment (ASP-HCl). Subsequently, ASP-HCl was subjected to a thermal pressure treatment (120°C, 0.1 MPa) for 15 min. The product was identified as avocado seed hydrolysed treatment 1 (ASH-1). The pH of ASH-1 was adjusted to 7.2 with a 10N NaOH solution (Karal, 2015). After that, ASH-1 was submitted to a second thermal pressure treatment in similar conditions (120°C, 0.1 MPa) for 15 min. The avocado seed hydrolysed treatment 2 (ASH-2) was stored at 4°C.

2.3 Experimental culture medium (ECM) preparation

Experimental culture medium (ECM) was formulated as follows: M9 minimal salts medium (1X concentration) modified as indicated in Maniatis *et al*., (1982), 1 mM $MgSO_4$ (high purity), 0.1 mM $CaCl_2$ (high purity), distilled water and volume required of ASH-2 to obtain 6.67, 13.33, and 20 g/L of reducing sugars (final concentration).

2.4 Morphology of hydrolysed treatments

The morphology was observed in an optical microscope Olympus BX50 (Olympus, Japan) at 40x/1.00 objective (U-Plan-Apochromat, Olympus, Japan) coupled to a digital camera Lumenera- Infinity

3 (Lumenera, Canada) with incandescent illumination and polarised light. The sizes of the images were 2560 x 1920 pixels captured as RGB colour format and stored in tagged image file format (.tiff). The image resolution was 0.29 pixels/μm^2. The analysed digital image (ADI) technique consisted in converting the original image (RGB) to 8-bits format (grey scale). Subsequently, the starch granules were cropped from the image in grey-scale by using the software Corel Paint Shop Pro Photo XI (V11.0, Corel Corporation, USA), and transferred into a new image with white background. This technique allowed viewing of segmented plant cell structures or biomaterials with different focal planes and diffraction of light (Sánchez-Segura *et al*., 2015). Subtracting the grey level binarized all images and a fill holes tool was applied. Calibration of the microscope's field sizes was performed through a stage micrometer (Nikon, OB1, Japan) under the same magnification and illumination conditions as the observed samples.

The analyzed treatments were (i) ASP-HCl, (ii) ASH-1, (iii) ASH-2, and (iv) ECM. The morphometric parameters of hydrolysed treatments were evaluated using Sigma Scan Pro software (V5.0, SPSS, USA). Descriptors analysed were area (A), perimeter (P), Feret´s diameter (Fd), and shape factor (Sf). Fd calculates the diameter of a fictitious circular object that has the same area as the object being measured according to Matusiewicz *et al*., (2007) (Eq. 1). Sf or *circularity* is based on the projected area of the granule (A) and the overall perimeter of the projection (P) according to Bouwman *et al*., (2004) (Eq. 2).

$$Fd = \sqrt{\frac{4A}{\pi}} \tag{1}$$

$$Sf_{circularity} = \frac{4.\pi.A}{P^2} \tag{2}$$

2.5 Particle size of avocado seed powder and hydrolysed treatments

The experiments were performed using a particle size analyzer CILAS 1090 (CILAS, France). The operation conditions were using deionized water as dispersing agent and static light scattering (angle of 45° with lasers 635 and 830 nm) for polydispersed samples in liquid mode. A sample (500 μL) was added in the liquid sample chamber and 49.5 mL of deionized water was used as carrier medium (dilution factor 1:100), according to Peralta *et al*., (2014).

2.6 Quantification of reducing sugars

Breaking of the starch granules during ASP hydrolysis process was quantified by measuring reducing sugars using 3,5 dinitrosalicylic acid (DNS) reagent (modified method from Miller, (1959)). DNS reagent was prepared with 10 g/L DNS (Sigma Aldrich, 2015), 1 g/L sodium sulfite (Karal, 2015), 2 g/L phenol (Merck, 2015), and 10 g/L sodium hydroxide (Karal, 2015). 1 mL of ASH-1 or -2 was diluted in distilled water (1:200) and mixed with 1 mL of DNS reagent. Samples were boiled (15 min) and maintained in ice bath. 8 mL of distilled water was then added and the absorbance at 575 nm was measured. The concentration of reducing sugars in the samples was determined using the calibration curve (0 to 1.0 g/L) of dextrose (Karal, 2015). Analyses were conducted in triplicate.

2.7 Bacterial growth conditions

Escherichia coli K12 MG1655 (ATCC 700926) was used as a model microorganism to evaluate the bacterial growth in ECM and LB medium. To determine the effect of nitrogen source on bacterial growth, the ECM was prepared with half the normal concentration of NH_4Cl (respect M9 medium) and NH_4Cl-free ASH-M9 salts medium. LB medium was prepared according to Sezonov *et al*., (2007). Precultures of *E. coli* K12 MG1655 were cultivated overnight in LB medium at 37°C with shaking at 170 rpm. These precultures were centrifuged for 20 min at 3000 rpm and washed with M9 minimal salts medium. Flasks containing ECM (30 mL) were inoculated with 3 mL of bacterial inoculum at 0.2 optical density (OD_{600nm}) units in a spectrophotometer (Beckman Coulter DU 640). After that, the cultures were incubated at 37°C with shaking conditions at 170 rpm. Bacterial growth was measured for 24 h, all measurements were made at OD_{600nm}. To calculate biomass production or dry cell weight (gDCW), the Akhtar and Jones (2009) method was used (Eq. 3).

$$1OD_{600} = 0.47gDCW/L \tag{3}$$

2.8 Bioreactor cultivation

E. coli colonies were cultivated in 250 mL shake flasks containing 100 mL of ECM. Precultures were grown overnight in an orbital shaker (37°C, 200 rpm). The precultures (50 mL) were taken during exponential growth phase (OD_{600nm}=2.0) and were used to inoculate a 3 L bioreactor. Growth assays were

performed in a stirred tank reactor (STR) with two six-blade Rushton turbines (Applikon, The Netherlands); the operation volume was 1.7 L. The pH and dissolved oxygen (DO) were monitored online using autoclavable sensors (AppliSense, The Netherlands). These parameters were automatically controlled using a PID control scheme (My-control system, Applikon). A 15% v/v NH_4OH solution was used to maintain pH at 7.00. A silicone-based antifoaming agent (VRF-30) was added. Gas flow rate was maintained at 2.5 vvm (volume of air per volume of medium per minute) in batch cultures. BioXpert software (Applikon) was used for data acquisition. In the case of ECM, two cultivations were conducted, at 150 rpm and 200 rpm. For the LB medium, the kinetic assay was performed at 150 rpm. On the one hand, bacterial growth was monitored in intervals of two hours for the first 16 hours and thereon every four hours (OD_{600nm} on a Beckman Coulter DU 640 spectrophotometer). On the other hand, biomass production was quantified in terms of dry cell weight (DCW). The samples were centrifuged at 3000 rpm for 20 min at 4°C. Pellets were subsequently dried in an oven at 80°C for 24 h (PRONALAB, México). Finally, the biomass produced was determined by gravimetric method.

2.9 Statistical analysis

Hydrolysed treatments of avocado seed powder (ASP-HCl, ASH-1, ASH-2, and ECM) were analysed in triplicate. Evaluations of morphometric features were performed on 100 granules of starch. Starch granules were segmented from 10 images captured by slice preparation for each hydrolysed treatment. The average ± standard deviation values for each morphometric parameter were reported. Particle sizes of hydrolysed treatments were measured in triplicate. Analysed replicas allowed for a typical error under 5% (Casas-Forero *et al*., 2011). Determination of reducing sugar concentration was conducted in triplicate. Data were evaluated by analysis of variance (one-way ANOVA) with $\alpha \leq 0.05$. Differences between means were determined by Tukey-Kramer Test, $\alpha \leq .05$ (NCSS, 2007).

3 Results and discussion

3.1 Morphometry and particle size of hydrolysed ASP

The main components of particles in the ASP were starch and fibrous material. According to analysis of the morphology and morphometry, the starch granules were oval-shaped with concentric rings (Fig. 1A, white arrows). Similar features were reported for potato tubers, and avocado (Kahn, 1987). We observed starch granules with an average A of 33.75μm^2 ± 12.95, P of 21.98 μm ± 4.48, Fd of 6.41 μm ± 1.34, and Sf of 0.84±0.04 (Table 1). The A/P ratio of the starch granules was 1.53, indicating that the structures had a larger perimeter in less area, i.e. irregular morphology (Fig. 1B, white arrow). The irregularity of the starch granules might be a result of the grinding method for extraction. Fd and Sf parameters supported this qualitative description of the oval or semi-spherical shape. Sf values of circularity were observed ranging from 0 to 1, where unity represents a perfect sphere and values near zero represent longer or rougher shapes (Bouwman *et al*., 2004). After hydrolysis (ASH-1 and ASH-2), the starch granules showed a diminution of A (1.61 μm^2 ± 0.67 to 0.31 μm^2 ± 0.18) and P (5.92 μm ± 1.66 to 2.35 μm ± 0.85). Similar morphometric changes were found in Fd values (1.40 ± 0.29 to 0.64 ± 0.17). A different behaviour was observed in Sf descriptor (0.58 ± 0.13 to 0.68 ± 0.14), indicating an increase in the sphericity of the residual starch. The A/P ratio of these granules was 0.27 for ASH-1 and 0.13 for ASH-2, this affectation was caused by hydrolyzed treatment in starch (de-polymerization of sugar molecules).

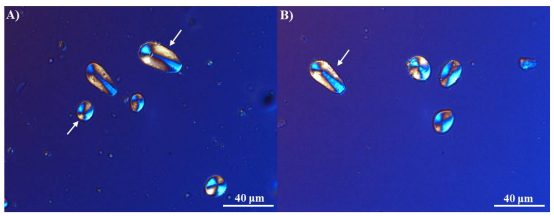

Fig. 1. Shapes of starch granules found in avocado seed powder. A) Starch granules with oval shape. B) Some modified starch granules with irregular morphology (white arrow).

Table 1. Morphometric features of the starch granules of avocado (*Persea americana* Mill.), during hydrolysis treatment.

	ASP-HCl	ASH-1	ASH-2	ECM
A (μm^2)	33.75 ± 12.95	1.61 ± 0.67	0.31 ± 0.18	0.03 ± 0.01
P (μm)	21.98 ± 4.48	5.92 ± 1.66	2.35 ± 0.85	0.76 ± 0.02
Fd (μm)	6.41 ± 1.34	1.40 ± 0.29	0.64 ± 0.17	0.21 ± 0.21
Sf	0.84 ± 0.04	0.58 ± 0.13	0.68 ± 0.14	0.84 ± 0.17

ASP-HCl: avocado seed powder with HCl; ASH-1: avocado seed hydrolyzed treatment one; ASH-2: avocado seed hydrolyzed treatment two; ECM: experimental culture medium. A: area, P: Perimeter, Fd: Feret´s diameter, Sf: Shape factor Average ± standard deviation values of each parameter were obtained to 100 measured particles segmented of 15 images of 3 experimental replicate.

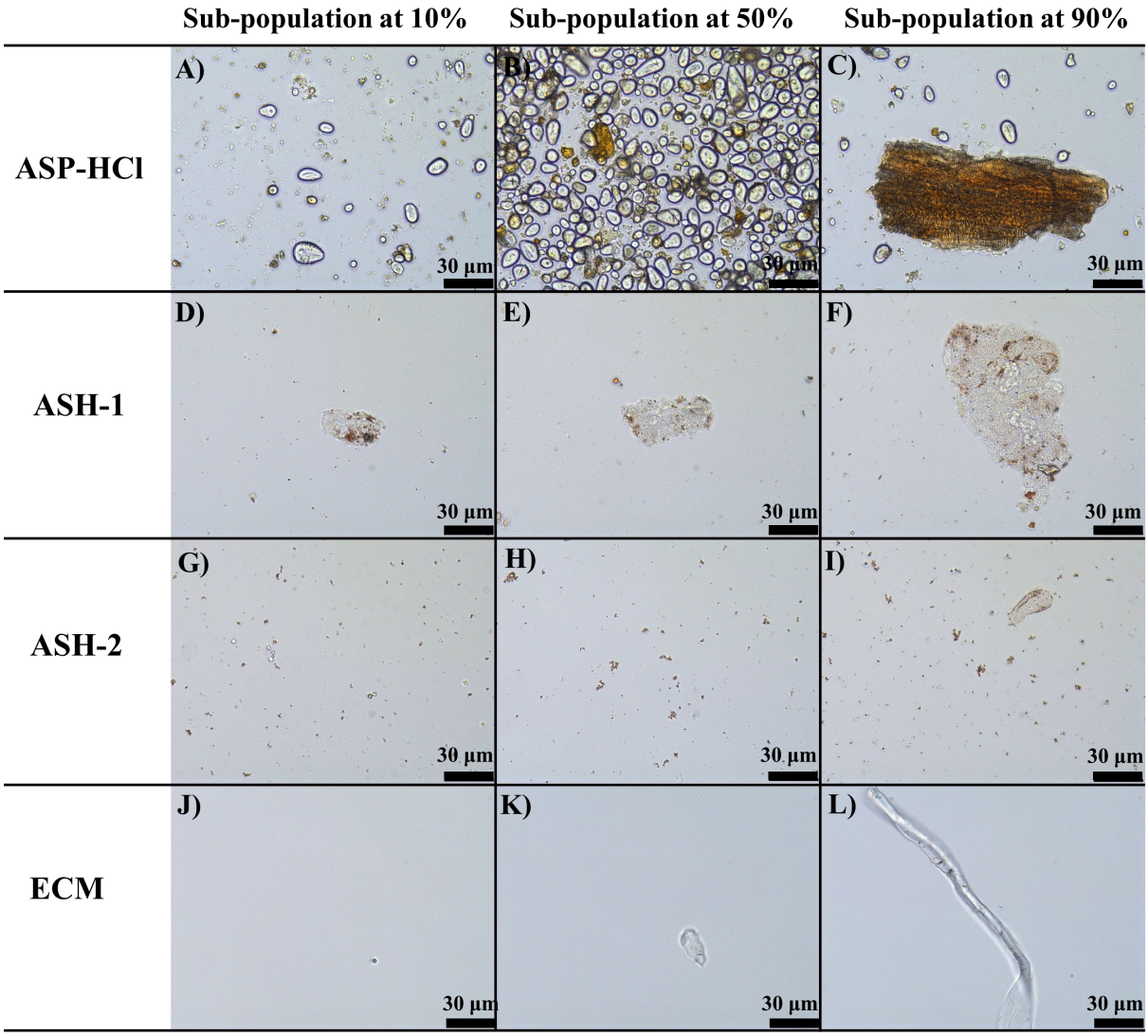

Fig. 2. Morphology of the particles of avocado seed powder showed at different sub-populations. (A-C) Avocado seed powder with HCl (ASP-HCl), (D-F) avocado seed hydrolysed treatment one (ASH-1), (G-I) avocado seed hydrolysed treatment two (ASH-2), and (J-L) experimental culture medium (ECM).

Table 2. Distribution of the particle size in the processes used on avocado seed powder. Avocado seed powder with HCl (ASP-HCl), avocado seed hydrolysed treatment one (ASH-1), avocado seed hydrolysed treatment two (ASH-2), and experimental culture medium (ECM).

	PARTICLE SIZE (μm)	DIAMETER 10%*	DIAMETER 50%**	DIAMETER 90%***
ASP-HCl	23.53	6.54	20.96	43.84
Std. Dev.	±0.27	±0.40	±0.22	±0.78
Typ. Err.	0.16	0.45	0.12	0.23
ASH-1	47.28	4.83	42.1	94.91
Std. Dev.	±16.84	±4.51	±13.17	±38.28
Typ. Err.	9.72	22.10	7.60	2.60
ASH-2	34.05	2.31	33.83	83.43
Std. Dev.	±24.20	±0.28	±13.28	±43.51
Typ. Err.	13.97	25.12	7.67	0.16
ECM	36.99	1.17	38.25	75.46
Std. Dev.	±24.47	±0.98	±21.16	±52.24
Typ. Err.	14.13	30.16	12.21	0.56

* Diameter at 10% of the 500μm of the maximum detection of CILAS ** Diameter at 50% of the 500μm of the maximum detection of CILAS *** Diameter at 90% of the 500μm of the maximum detection of CILAS ASP-HCL: avocado seed powder with HCl; ASH-1: avocado seed hydrolysed treatment one; ASH-2: avocado seed hydrolysed treatment two; ECM: experimental culture medium. Average ± standard deviation and typical error values of each measured were obtained of 3 experimental replicate.

The major loss of spherical shape is shown in the ASH-1 (Table 1). Finally, in the ECM, the A and P of measured particles did not show starch granules residues. The A/P ratio was 0.03, which is similar to A, whilst Sf was similar to the initial shape of starch (Table 1).

The particle sizes of the ASP are shown in Table 2 and the morphologies are shown in Fig. 2. The order of the images in Fig. 2 is ASP-HCl (Figs. 2A-C), ASH-1 (Figs. 2D-F), ASH-2 (Figs. 2G-I), and ECM (Figs. 2J-L). The average values between treatments indicate a tendency for increased size of particles of the seed powder during the thermal-pressure hydrolysed treatments (Table 2). However, the sub-population at 10% diameter (50 μm) of the measured particles indicates a different tendency since the sizes of particles in the ASP-HCl were below 6.54 μm. These measurements were comparable to those observed in micrographs (Fig. 2A). A similar tendency was observed for ASH-1 (Fig. 2D) and ASH-2 (Fig. 2G) whereas ECM produced particles with a size of 1.17 μm (Fig. 2J).

The sub-population at 50% diameter (250 μm) of the measured particles displayed a reduction of the particle size during the hydrolysis processes. The size of particles in the ASP-HCl was below 20.96 μm (Table 2). This dimension corresponded to starch granules of avocado seed (Fig. 2B). After the first hydrolysis treatment (ASH-1), the major part of the observed particles was composed of fibrous material and aggregates of residual starch, but complete starch grains were not observed (Fig. 2E). After the second hydrolysis (ASH-2), the size and presence (Fig. 2H) of particles decreased (Table 2) in the hydrolysed medium; however, the particles in the ECM correspond to fibrous material (Fig. 2K).

In the sub-population at 90% diameter (450 μm) an increase in the size of particles measured was observed due to the formation of agglomerates from the insoluble fraction of the avocado seed powder. The sizes of particles in the ASP-HCl were below 43.84 μm (Table 2). These sizes correspond to the fractions of the pulp, which is mainly composed of vessel elements and fibrous material (Fig. 2C). After the first hydrolysis treatment (ASH-1), the major portion of observed particles was composed of fibrous material; also delignification was observed (Fig. 2F). After the second hydrolysis (ASH-2), the size and presence of particles decreased (Table 2) in the medium (Fig. 2I). The particles of the ECM correspond to the fibrous material without lignin (Fig. 2L).

The hydrolysis process produces residual fibrous material formed by the amorphous cellulose fraction, and crystalline regions or microcrystalline cellulose

(MCC) (Adel *et al*., 2011). According to previous reports, acid hydrolysis is the conventional method for manufacturing MCC. For example, Bolio-López *et al*., (2011) obtained cellulose from agro-industrial waste banana through conventional chemical methods such as acid hydrolysis, chlorination, alkaline extraction, and bleaching. MCC crystals form particles of about 15 ± 20 μm diameters. These particles can form aggregates with a size of 20 to 200 μm (Picker-Freyer, 2007). Aggregates having this description were found in ASH-2 (Table 2) and morphological descriptions were similar to ASH-1 and ASH-2 observations (Fig. 2D-2I). On the other hand, ADI technique was the best technique for monitoring the degradation of starch granules (size and shape), while the static light scattering measurements allowed us to establish the size of the aggregates formed during the hydrolysing treatments.

3.2 Acid-autoclave hydrolysis treatment of avocado seed powder

In order to investigate breaking of the starch granules during the ASP hydrolysis processes, the amount of reducing sugars was measured in the various treatments. As shown in Fig. 3, the concentration of reducing sugars for ASP-HCl turned out to be 9.5 ± 0.4 g/L while the level of soluble reducing sugars notably increased to 137 ± 6 g/L (ASH-1) and 140 ± 9 g/L (ASH-2) after the hydrolysis treatments. The main chemical components of avocado seeds have been reported to be (in wet basis): moisture around 50 %, ash 1.24-1.34 %, protein 2.38-2.45 %, total sugar 2.21-3.50 %, starch 27.54-29.80 %, crude fibre 3.65-4.14 %, and 7.76-9.25 % of undetermined materials (Weatherby and Sorber, 1931; Lacerda *et al*., 2014). The main component is the starch. Different pre-treatments (physical, chemical, and enzymatic) have been reported to increase starch decomposition, through the breaking down of amylose and amylopectin chains. For example, the following pre-treatments have been used extensively: alkaline, concentrated and diluted acid hydrolysis, and steam explosion (Kobayashi *et al*., 1998; Jayakody and Hoover, 2002; Aguilar-Rivera and Canizales-Leal, 2004; Wang *et al*., 2012). In this work, acid-autoclave treatment of ASP was conducted. In this method generated pressure causes explosion of starch granules, making the starch polymers more accessible to the action of acid for hydrolysis. If the treatment results in the decomposition of sugar polymers to less complex carbohydrates, then nutrients will be available. In this work, the breaking of starch granules was evidenced by the increased concentration of reducing sugars in ASH-1 (137 ± 6 g/L) and ASH-2 (140 ± 9 g/L) compared with ASP-HCl (9.5 ± 0.4 g/L). Accordingly, the hydrolysed starch contained in the ASH-2 can be used as the main carbon source of ECM for *E. coli* growth.

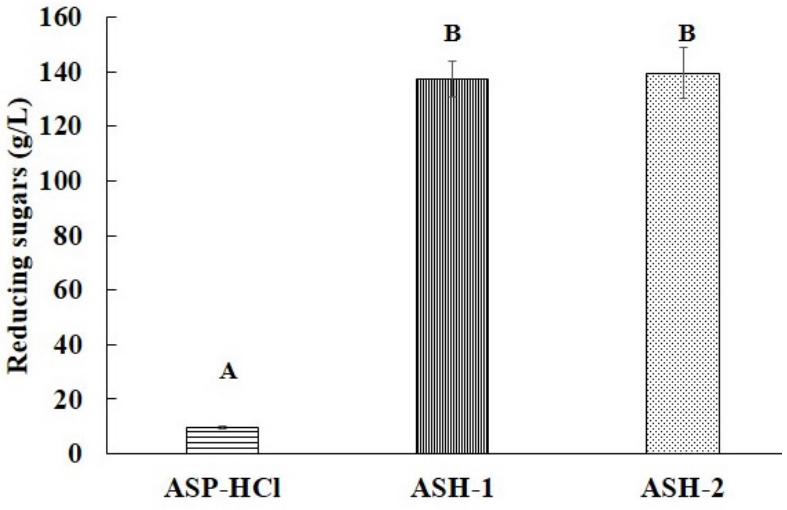

Fig. 3. Reducing sugars concentration during acid-autoclave hydrolysis of avocado seed powder. Avocado seed powder with HCl (ASP-HCl), avocado seed hydrolysed treatment one (ASH-1), avocado seed hydrolysed treatment two (ASH-2). Means with different letters are significantly different. Tukey-Kramer Test, $\alpha \leq 0.05$, (NCSS, 2007).

Table 3. Assays of *E. coli* growth using the ECM at different concentrations of reducing sugars. Biomass production and biomass yield were compared with LB medium.

Reducing sugar concentration (g/L)	Corrected OD_{600}	Biomass (g/L, dry basis)	Yield (gBiomass/ gSP)
6.67	2.27	1.10	0.33
13.33	3.72	1.75	0.27
20	4.73	2.22	0.23
15*	2.90	1.36	0.09

*Assuming that LB medium contains 10 g/L of peptone and 5 g/L of yeast extract

Table 4. Evaluation of ECM supplemented with NH_4Cl as nitrogen source to support *E. coli* growth. Biomass production and biomass yield were compared with ASH (NH_4Cl-free).

NH_4Cl (mM)	ASH (% v/v)	Corrected OD_{600}	Biomass (g/L, dry basis)	Theoretical Biomass (g/L, dry basis)	Yield (gBiomass/ gSP)
18.6	10	3.95	1.86	1.93	0.28
9.35	10	2.53	1.19	1.02	0.18
0 *	10	0.24	0.11	0.00	0.02

* ASH as the sole nitrogen source (NH_4Cl-free)

3.3 Effect of reducing sugars concentration and nitrogen supplementation on E. coli growth

The effect of the reducing sugars concentration in ECM was measured in terms of biomass production and biomass yield of *E. coli* at 24 h. Using ECM with 6.67 g/L of reducing sugars, a biomass production of 1.0745 gDCW/ L was reached, whereas using ECM with 13.33 and 20 g/L of reducing sugars resulted in biomass of 1.75 and 2.22 gDCW/L, respectively (Table 3). The biomass yield was 2.5-fold higher in the ECM than LB medium (0.23 vs 0.09) (Table 3). According to an amino acid analysis, avocado seed contained 0.67 mg/g amino acids and around 11% of unknown components that might contain nitrogen, which constitutes 14% of the total cell weight of *E. coli*, (Neidhart *et al*., 1990).

Nitrogen sources are usually the most expensive components in the elaboration of culture media (Taskin and Kurbanoglu, 2011). To determine whether pre-treated avocado seed could supply, at least partially, the nitrogen source in the medium, we assayed the ability of *E. coli* to grow in ECM with half the normal NH_4Cl concentration (18.6 mM) and in NH_4Cl-free ECM. As shown in Table 4, the growth of *E. coli* was lower in ECM with reduced or no NH_4Cl. Half the normal concentration of NH_4Cl (9.35 mM) resulted in a 1.6-fold decrease in biomass yield compared with that achieved using medium with normal concentrations of NH_4Cl (0.18 and 0.29 g DCW/ g SP, respectively). The amount of nitrogen in ECM is approximately 7.18 mM (assuming the average molecular weight of amino acids is 118.9 g/mol (Hachiya *et al*., 2007) and that the amount of amino acids present in hydrolysed seed is 0.67 mg/g). The biomass yield obtained using ECM was related with theoretical biomass yield to *E. coli* growth. This might be due to the contribution of other nitrogenated compounds present in the ECM. Hence, hydrolysed avocado seed did not supply sufficient nitrogen compounds to support *E. coli* growth, thus requiring an additional nitrogen source. Nevertheless, inorganic sources of nitrogen are still cheaper than organic sources, such as the casein peptone used in LB (Aspmo *et al*., 2005).

3.4 Growth of E. coli in bioreactor

Growth kinetic studies of *E. coli* were carried out in a bioreactor using optimal conditions observed at flask level. For the ECM, 20 g/L of the reducing sugar concentration were used in two fermentations, at 150 rpm and 200 rpm. The growth of *E. coli* in ECM increased as a function of agitation velocity (Figs. 4A and 4B). After 12 h, maximum growth was reached at 200 rpm (2.25 gDCW/L) whereas growth was lower at 150 rpm (1.92 gDCW/L). In contrast, studies in flask showed maximum growth after 24 h of cultivation using 20 g/L of reducing sugars.

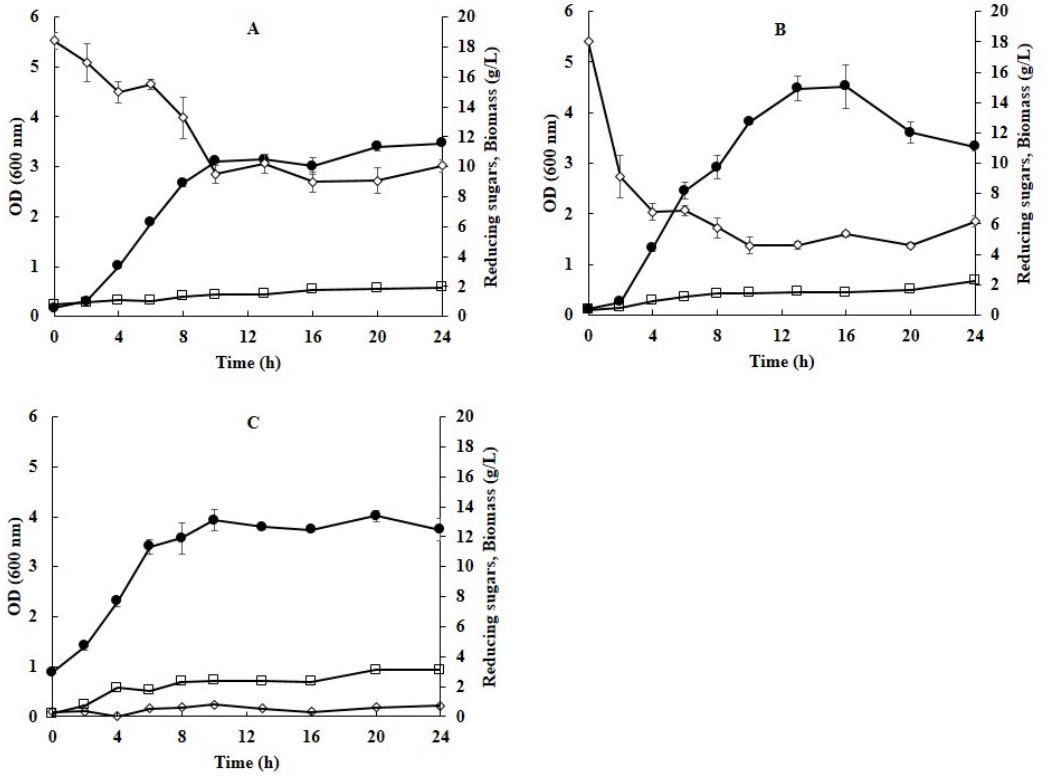

Fig. 4. Growth kinetics of *E. coli* strain K12 MG1655 in different culture media in a controlled bioreactor. A) ECM, 150 rpm; B) ECM, 200 rpm; C) LB medium, 150 rpm. Variables: optical density at 600 nm (−−); Reducing sugars (− ⋄ −); Biomass (−□−); Variables: optical density at 600 nm (− · −).

A quick depletion of reducing sugars was also observed in the ECM at 200 rpm (Fig. 4B), reaching a residual concentration of 5.33 g/L. As a consequence, the growth rate was higher at the agitation rate of 200 rpm (μ= 0.44 h^{-1}) than at 150 rpm (μ= 0.36 h^{-1}). For LB medium, the kinetic assay was performed at 150 rpm (Fig. 4C). Bacterial growth was 2-fold higher in the bioreactor (3.10 gDCW/L) than in flask (1.36 gDCW/L) and a growth rate of 0.37 h^{-1} was reached. However, the concentration of reducing sugars was maintained below 0.5 g/L (Fig. 4C). According to previous reports, the LB medium contains a low concentration of sugars, which are depleted during an initial phase of growth; after that, amino acids are used as a carbon source (Sezonov *et al*., 2007). For both media (ECM and LB), bacterial growth increased due to the operating conditions of the bioreactor (aeration and mixing). Stirred tank bioreactors provide high rates of mass and heat, and excellent mixing (García-Ochoa and Gómez, 2009). In these systems, many variables affect the mass transfer and mixing, but the most important among them are stirrer speed, type and number of stirrers, and gas flow rate (García-Ochoa and Gómez, 2009; Raffo-Durán *et al*., 2014).

Different studies have used waste materials as culture medium (Andualem and Gessesse, 2013; Famurewa and David, 2008). The simplicity of treatment reported in the present study suggests that avocado seeds are a cheap and sustainable culture medium for growth of *E. coli* and other strains of interest in biotechnological processes.

Conclusion

An efficient methodology was established to formulate an experimental culture medium (ECM). The hydrolysis treatments of ASP produced a reduction of particle size of starch granules. Reduction of particle size (33.75±12.95 μm^2 at 0.03±0.01 μm^2) was correlated with increasing of reducing sugars in ECM for bacterial growth. Additionally, the ADI technique was more sensitive compared to static light scattering to evaluate reduction of starch granules. Furthermore, the concentration of reducing sugars

in the ECM affected the biomass production of *E. coli* (20 g/L of reducing sugars produced 2.22 gDCW/L) compared with LB medium (1.36 gDCW/L) in flasks. In contrast, studies in bioreactor showed an increase of biomass production (3.10 gDCW/L), 2.28 fold with respect to flasks. Hence, avocado seed waste represents a valuable raw material for the development of culture medium to grow and maintain bacteria for biotechnological applications. Additionally, we propose treatment of avocado seed waste using hydrolysis as a new method suitable for the disposal of residues produced by the agronomic and food process industries.

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Nomenclature

A	area
ASH	avocado seed hydrolysate
ASP	avocado seed powder
cv.	cultivar
DNS	dinitrosalicylic acid
FAO	Food and Agriculture Organization
Fd	Feret's diameter
OD_{600}	optical density at 600 nm
P	perimeter
Sf	shape factor
SP	seed powder

Greek symbols

μ	growth rate
α	significance level
π	ratio between length of a circle and its diameter

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