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# ANTIOXIDANTS AND BIOMETHANE PRODUCTION FROM *Opuntia* VARIETIES

Obtención de antioxidantes y producción de biometano a partir de variedades de *Opuntia*

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Key words: anaerobic digestion, flavonoids, nopal, *Opuntia engelmannii*, *Opuntia ficus-indica*, phenolic compounds.

## ABSTRACT

In this study, *Opuntia* varieties were investigated for their potential to fit a biorefinery approach. This work proposed the extraction of antioxidants and the production of biomethane from their solid effluents. Three Opuntia varieties –*O. ficus-indica* var. Milpa Alta (OfiM), *O. ficus-indica* var. Copena (OfiC) and *O. engelmannii* (Oe)– were subjected to solvent extraction. Our results revealed that 80% methanol yielded the highest amount of phenolic compounds (8.99 mg/g) and flavonoids (1.67 mg/g) in OfiM. Subsequently, biomethane potential experiments compared raw and residual biomasses, as well as inoculum-to-substrate ratio (ISR, 0.5, 1.5 and 2.5). Biomethane yields were lower with residual biomass compared to raw *Opuntia*. The highest biomethane yield of 552 mL $CH_4$/g VS was achieved with raw OfiM at ISR 2.5. For the second assay of OfiM, biomethane yields were 412 and 458 mL $CH_4$/g VS, respectively for residual and raw biomasses. Despite a decrease of approximately 10% in biomethane yield due to extraction operation, the coupling of both processes becomes highly desirable to obtain high-value extractable compounds and substantial biomethane production, which is a step towards the *Opuntia* biorefinery.

Palabras clave: digestión anaerobia, flavonoides, nopal, *Opuntia engelmannii*, *Opuntia ficus-indica*, compuestos fenólicos.

## RESUMEN

En este trabajo, se investigaron variedades de *Opuntia* para comprobar su potencial para encajar en un enfoque de biorrefinería. Para ello, se propuso la extracción de antioxidantes y la producción de biometano a partir de sus efluentes sólidos. Tres variedades de *Opuntia* –*O. ficus-indica* var. Milpa Alta (OfiM), *O. ficus-indica* var. Copena (OfiC) y *O. engelmanii* (Oe)– se sometieron a extracción con disolventes. Se encontró que el metanol al 80 % produjo la mayor cantidad de compuestos fenólicos (8.99 mg/g) y flavonoides (1.67 mg/g) en OfiM. Posteriormente, en los experimentos de potencial de biometano se compararon biomasas crudas y residuales, así como la relación inóculo-sustrato (ISR, 0.5, 1.5 y 2.5). Los rendimientos de biometano fueron menores con biomasa residual que con *Opuntia* cruda. El mayor rendimiento de biometano de 552 mL $CH_4$/g SV se alcanzó con OfiM cruda y ISR 2.5. En el segundo ensayo de OfiM, los rendimientos de biometano fueron de 412 y 458 mL $CH_4$/g SV para biomasas residuales y crudas, respectivamente. A pesar de una disminución de aproximadamente 10 % en el rendimiento de biometano debido al proceso de extracción de fenólicos, el acoplamiento de ambos procesos resulta conveniente para la obtención compuestos extraíbles de alto valor y una producción sustancial de biometano, lo que representa un paso hacia la biorrefinería de *Opuntia*.


## INTRODUCTION

*Opuntia ficus-indica* is the representative species of its genus due to its edible traits and importance. It is known as "nopal" in Spanish, and prickly pear cactus in English. Nopal holds significant cultural importance as a symbol of Mexico's identity. From gastronomy to archaeology, *nopales* are present in popular dishes, on the national flag and even in the legendary myth of the founding of the great *Tenochtitlan* (Inglese et al. 2018). *Opuntia* or nopal (from *nopalli* in Nahuatl) is a perennial crop that enters its productive stage two years after planting, reaches its potential at five years and has a production period of at least 20 years. These plants can be arborescent, shrubby or creeping, with or without a well-defined trunk, and their flattened leaves (known as cladodes or stalks) can be oblong, elliptical, obovate, subcircular or circular. The spines on the nopales do not have a sheath, and their flowers can grow up to 6 cm long, commonly yellow, changing color on the second day due to the change in pH (Brahmi et al. 2022). Nopales produce fruits of different shapes, with thin or thick walls, and can be sweet, sour or bittersweet (Scheinvar, Olalde Parra, and Sule 2011).

*Opuntia* genus embraces a great variety of Cactaceae plants native to Mexico and parts of the southwestern United States. Scheinvar et al. (2011) recognized 93 wild species and 15 varieties and/or subspecies of *Opuntia* from Mexico, 90% being endemic; thus, it has been stated that Mexico is its country of origin. The nopal cactus has oval-shaped pads, or cladodes, used as a vegetable in Mexican cuisine with nutritional and medicinal properties. Nopal as a crop presents high yields and low water requirements; it is a versatile plant resistant to harsh weather conditions. Species and varieties of nopal have been studied due to their potential as raw material for obtaining antioxidant compounds, such as phenolic acids and flavonoids. The most abundant compounds in *Opuntia* spp. are quercetin, kaempferol and isorhamnetin (De Santiago et al. 2021), whose use in pharmaceutical and nutraceutical industries is being investigated (Madrigal-Santillán et al. 2022).

Antioxidants serve as a defense system in the body, avoiding or diminishing the damage by reactive oxygen species normally produced during physiological processes. They can be either endogenous, produced within the body, or exogenous, obtained from food. Due to concerns regarding the safety of synthetic antioxidants associated with allergies, carcinogenicity, and DNA abnormalities, current research focuses on exploring natural and cost-effective sources of antioxidants (Shahidi and Ambigaipalan 2015).

Flavonoids are secondary plant metabolites with a polyphenolic structure; they have various positive biochemical and antioxidant effects associated with degenerative diseases. The extraction of phenolic compounds (especially flavonoids) has been extensively studied for medicinal plants and *Opuntia* species have also been studied in this context, with an emphasis on antioxidant and antibacterial activities. While many articles focus on evaluating the total antioxidant activity of various parts of the plant, such as the cladode, the prickly pear, or the flower, others aim to obtain extracts with high concentrations of phenolic compounds and flavonoids, often intended for use as food additives or in the development of functional foods.

De Santiago et al. (2020) studied the polyphenolic compounds of *O. ficus-indica* cladodes while evaluating four methods: water, ethanol 80%, pure ethanol, and successive extractions of methanol 50%, acetone 70% and water. The use of solvents with different polarities has resulted advantageous for the extraction of flavonoids.

Ávila-Nava et al. (2014) compared the antioxidant activity of other foods with nopal cladodes; coffee (2.3 mmol trolox/g sample) and garlic (1.09 mmol trolox/g sample) ranked first and second place just before *Opuntia* (0.65 mmol trolox/g sample) and after plum (0.13 mmol trolox/g sample) and chia seeds (0.05 mmol trolox/g sample).

Aruwa et al. have been studying *O. ficus-indica* activities and properties, from fruit pulp and peels (Aruwa et al. 2019b) to cladodes (Aruwa et al. 2019a). They concluded that nopal has many macromolecular antioxidants encapsulated in the commonly extracted material since hydrolysis of the extracts showed greater antibacterial and antioxidant activities, being mostly glycosylated flavonoids. Santos-Zea et al. (2011) described this as well; they evaluated varieties of *Opuntia* species and identified glycosidic forms of isorhamnetin and kaempferol, which are flavonols usually found in nopal. As read in most of the references, flavonoid extraction methods from *Opuntia* carries out in 2 h extraction time, solid-to-liquid ratio of 1/10, and the methanol, acetone and water sequential extraction.

On the other hand, anaerobic digestion is a well-known process, also applied to *Opuntia* species. Sosa-López and Aké Madera (2017) patented the description of the process and equipment necessary for anaerobic digestion of *Opuntia*, considering cacti diluted from 3 to 5% in water. In the last five years, laboratory works have focused on batch production with co-substrates, mainly cow manure, to correctly balance the C:N ratio due to *Opuntia* being mostly carbohydrates. Also, pretreating methods to hydrolyze pectin and matrix carbohydrates have been studied. Calabrò et al. (2018) reported that methane production using *Opuntia ficus-indica* increased over raw biomass when the cladodes were pretreated with hydrochloric acid (20%, 80 °C). Interestingly, their results were above the theoretical maximum for glucose (373.25 mL $CH_4$/g VS), even for raw biomass. However, no convincing explanation was given. Moderate results were obtained by Valenti et al. (2018), for whom the best biomethane production (243.6 mL/g VS and 63.4% of methane) was achieved with a mixture of 20% citrus pulp, 15% sainfoin silage, 10% poultry manure, 15% *Opuntia* cladodes and 40% olive pomace, according to the use of agricultural by-products and residues from southern Italy.

Regarding the significance of CAM plants nowadays, they are promissory for exploiting poor and marginalized soils, as well as in areas where traditional crops do not thrive, due to their unique metabolism that efficiently utilizes water and nutrients. Lueangwattanapong et al. (2020) studied the biomethane potential of five CAM plants (*Ananas comosus, Agave angustifolia, Opuntia fragilis, Kalanchoe daigremontiana,* and *Euphorbia virosa*). The yield of *Opuntia* was 300.8 mL/g VS, the third best of the CAM plants, just behind *Kalanchoe* (337.6 mL/g VS) and *Agave* (381.7 mL/g VS).

Our group has previously worked with *Opuntia*; Ávila-Lara (2019) obtained succinic acid from *O. ficus-indica* pretreated chemically and enzymatically. On the other hand, Villela-Buenrostro (2018) applied life cycle analysis for electricity generation from nopal by producing methane using anaerobic digestion.

Combining bioproducts extraction and anaerobic digestion of biomass suits the biorefinery paradigm, which regarding to the use of *Opuntia* as a substrate has not been sufficiently or at all explored. This work aimed to develop a sequential processing for the valorization of *Opuntia*, extracting antioxidants and producing biomethane from the residual biomass. Antioxidant extraction capabilities of methanol and ethanol were evaluated at different concentrations on *O. ficus-indica* var. Milpa Alta, *O. ficus-indica* var. Copena, and *O. engelmannii*. The biomethane potential of raw and residual biomasses from the extraction was determined by varying the inoculum to substrate ratios.

## MATERIALS AND METHODS

### *Opuntia* recollection and preparation

Three *Opuntia* varieties were evaluated: edible *Opuntia ficus-indica* (OfiM) acquired from a local market in Monterrey, Nuevo Leon, Mexico; *Opuntia engelmannii* (Oe) sampled from the surroundings of the Parque de Investigación e Innovación Tecnológica, in Apodaca, Nuevo Leon; and *Opuntia ficus-indica* var. Copena (OfiC) retrieved from a plantation in General Cepeda, Coahuila, Mexico (N25°23'0" O101°27'13", altitude 1460 masl), in autumn (October 30) between 8 and 9 A.M. six-month-old cladodes of good quality without blemishes (with no plague, cuts or scars, deformities), which had not borne fruit, were selected.

Two cladodes were collected from each sampled plant, each 5 plants in the same direction of the furrow and 5 plants in the perpendicular direction, all of the same height and stalks of equal sizes (*i.e.* 20 cm long by 15 cm wide) (du Toit et al. 2018). In total,

20 kg were collected. Within 24 h after collection, the spines were removed from the pads, washed with tap water, disinfected with 70% industrial-grade ethanol, cut into squares, and liquefied. The liquid mass was placed in glass trays and allowed to dry at ambient temperature for 3 to 5 days. Dried biomass was ground in a coffee grinder to reduce particle size by less than 210 microns. Finally, the biomass was stored hermetically at a temperature below 20 ºC (Carrillo-Verástegui et al. 2022).

**Extraction procedure and design of experiments**

The extraction procedure consists of a 3x2x2 general factorial design to evaluate three factors: *Opuntia* varieties (OfiM, Oe, or OfiC), organic solvent (methanol or ethanol) and solvent concentration (80 and 100%). The response variables were total phenolic compounds, total flavonoids, antioxidant activity by scavenging DPPH free radicals, and extraction yield.

Extraction set-up begins with the placement of *Opuntia* biomass in serological bottles with the corresponding solvent at a solids-solvent ratio 0.1 g/mL (5 g biomass and 50 mL solvent). Afterward, suspensions were shaken in an orbital incubator at 150 rpm and room temperature (23.5 ± 1 °C) for 1 h (De Santiago et al. 2021). When finished, the suspension was centrifuged at 10,000 rpm for 15 min, and the supernatant was filtered through Whatman No. 1 paper and stored in Corning tubes for analysis. The sediments or residual solids were used for biogas production assays.

**Inoculum preparation**

Fresh inoculum for the biomethane potential assays was obtained from anaerobic sludge from a lab-scale anaerobic digester mounted following the Poggi-Varaldo method (Escamilla-Alvarado et al. 2012) and operated according to Rodríguez-Valderrama et al. (2020); it was fed weekly with vegetable and fruit residues at a mass retention time of 60 d. The inoculum was characterized as 9.99% of total solids, 33.68% of volatile solids on dry basis, pH 8.06.

**Biomethane potential assays**

Three sets of experiments were established to evaluate biomethane potential using *Opuntia* biomass. First, a 3x2 factorial design was used to evaluate the effect of *Opuntia* biomass (OfiM and Oe) and ISR (0.5, 1.5 and 2.5) on biomethane production.

The second experimental design was a unifactorial experiment where solid extraction residues from OfiC were evaluated at three ISR (0.5, 1.5 and 2.5). Finally, after choosing the best ISR for biomethane production, raw biomass was compared to the extract residues of all three *Opuntia* types. Residues were identified by adding an r before the *Opuntia* abbreviation, i.e., r-OfiM, r-Oe and r-OfiC.

The assays were carried out in duplicates in 124 mL serological bottles, with a constant operating volume at 50 mL. Substrates were added to the serological bottles after the inoculum in quantities according to the ISR proposed in the experimental designs; a control assay was prepared with only inoculum. The bottles were sealed with a butyl cap and an aluminum seal. The assays were incubated at 35 °C in an incubator (PRENDO HS-46, Mexico). The bottles were stirred manually daily, and methane production was measured using NaOH solution displacement method (Carrillo-Verástegui et al. 2022).

Maximum biomethane production and yields were obtained by fitting the data to the first-order model equation (Eq. 1):

$$B(t) = B_0(1 - e^{-kt}) \quad (1)$$

where, $B(t)$ is the biomethane yield (mL $CH_4$/g VS) at a given experimental time, $B_0$ is maximum biomethane yield (mL $CH_4$/g VS), $k$ is the rate constant (1/d) and $t$ is time (d). Biomethane production was calculated by the difference between the assays production for each substrate and the production of the control.

Overall experimental yields of biomethane were calculated on substrate VS basis according to Eq. 2:

$$B_{exp} = \frac{b_{exp}}{m_{sVS}} \quad (2)$$

where $B_{exp}$ is the experimental biomethane yield (mL $CH_4$/g VS), $b_{exp}$ is experimental biomethane production (mL $CH_4$) and $m_{sVS}$ is the mass of the added substrate in volatile solids (g VS).

Boyle equation (Eq. 3) was used to calculate the theoretical methane yield ($B_{0\text{-}Th}$; mL $CH_4$/g VS) from *Opuntia* empirical formulae ($C_{29}H_{60}O_{32}N$):

$$B_{0-Th} = \frac{22400\left(\frac{a}{2}+\frac{b}{8}-\frac{c}{4}-\frac{3d}{8}-\frac{e}{4}\right)}{12a+b+16c+14d+32e} \quad (3)$$

**Experimental analysis and characterization**

The inoculum and substrates were characterized by pH, solids profile, alkalinity, and volatile fatty acids. Solids profile, that is, total solids (TS) and

volatile solids (VS), were determined by gravimetry as described in APHA-AWWA-WEF (2017). Alkalinity and volatile fatty acids (VFAs) were measured by a titration method with two endpoints (5.75 for carbonate alkalinity and 4.3 for VFAs alkalinity) (Pérez and Torres 2008; Anderson and Yang 1992).

The extracts were characterized by TPC, TFC, antioxidant activity DPPH and yield. Total phenolic compounds were quantified using Folin-Ciocalteu reagent (Kechebar et al. 2017); the method was modified as follows: 125 µL of diluted sample was added to 500 µL of distilled water and 125 µL of Folin-Ciocalteu reagent; then, the mixture was shaken in the vortex and then added 1 mL of $Na_2CO_3$ 7% and diluted with 1 mL of distilled water. Samples were incubated for 90 min in the dark, and the absorbance was read at 760 nm. Gallic acid was used as a standard.

Flavonoid compounds were quantified using the $AlCl_3$ method (Pawar and Dasgupta 2018); briefly, 50 µL of the diluted sample were mixed with 10 µL of $AlCl_3$ 10%, 10 µL of sodium acetate 1 M and 150 µL of ethanol 95%. Samples were incubated for 40 min at room temperature in the dark. The absorbance was read at 415 nm, and quercetin was used as a standard.

Yield of extraction was calculated by letting a known volume evaporate and weighting the dried solid extract. Yield was calculated by:

$$Y_{ext} = \frac{W_{de}}{V_S} \quad (4)$$

where $Y_{ext}$ is the extraction yield in mg/mL, $W_{de}$ is the weight in mg of the extract after drying at 60 °C for 12 h and Vs is the volume of the sample to be dried.

For antioxidant activity, a DPPH microplate method was applied (Bobo-García et al. 2015). Briefly, 20 µL of diluted sample was mixed with 180 µL of a 150 µM DPPH solution in methanol 80%. Samples were incubated for 40 min at room temperature in dark, and the absorbance was read at 515 nm. DPPH scavenging was calculated as a percentage:

$$\%DPPH\ scavenged = \left(1 - \frac{A_{sample} - A_{blank}}{A_{sample} - A_{blank}}\right) x100 \quad (5)$$

**Statistical analysis**

Methane production data were fitted to first-order model (Equation 1) using SigmaPlot v.11 (SYSTAT software). In the extraction of phenolic compounds, the main effects for each factor were calculated from the average of the treatments at each level. The error ascribed to the main effects is the experimental design error, obtained from the square root of the quotient of the mean square of the error and the number of experimental repetitions (Montgomery 2017).

## RESULTS AND DISCUSSION

**Antioxidant compounds extraction**

**Figure 1** shows the main effects of the factors (*Opuntia* variety, organic solvent and concentration) on the phenolic compound's extraction. There were four responses on this assay: phenolic compounds (**Fig. 1A**), flavonoid compounds (**Fig. 1B**), extraction yield (**Fig. 1C**) and antioxidant activity DPPH scavenging (**Fig. 1D**). The most important observation was that the 80% methanol assays allowed for the best phenolic and flavonoid compounds extraction, and OfiM was the best substrate of *Opuntia*. The results according to the concentration were similar in all responses (**Figs. 1A** and **1B**). It was observed that, regardless of the *Opuntia* variety and organic solvent, the 80% concentrations gave higher yields, TPC and TFC, and higher antioxidant activity than the 100% concentration. Considering that flavonoids are phenolic compounds, the factors behaved similarly (**Figs. 1A** and **1B**). The highest values obtained were 8.99 mg GAE/g and 1.67 mg QE/g (**Table SI**).

The high concentrations of phenolics and flavonoids using methanol and aqueous ethanol may be because the extracted compounds contain sugars in their structures; that means that the flavonoids that were extracted may be glycosylated flavonoids (Santos-Zea, Gutiérrez-Uribe, and Serna-Saldivar 2011; Antunes-Ricardo et al. 2020).

In **figures 1C** and **1D**, the main effects of the factors on extraction yield and DPPH scavenging show similarities. The pattern of the best results for extraction with 80% methanol is maintained. In the case of extraction yield, the interaction of the type of solvent with its concentration was not significant ($p > 0.1321$, **Table SIV**). For DPPH radicals scavenging, type of nopal was not a significative factor ($p > 0.1332$, **Table SV**), which can be observed in the main effects graph (**Fig. 1D**).

However, a higher phenolic and flavonoid compound concentration did not ensure a higher DPPH scavenging; then, there could be another important antioxidant compounds that enhance DPPH antioxidant activity in Oe extract, for example.

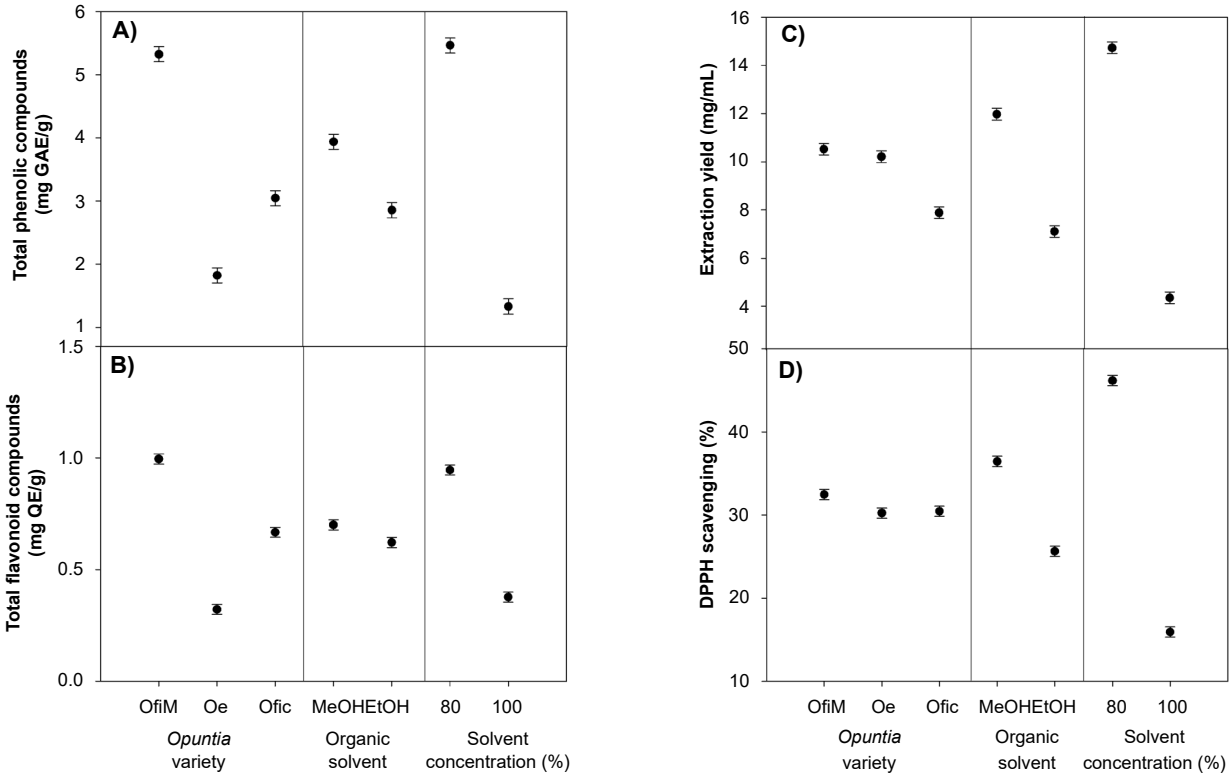


**Fig. 1.** Main effects of the 3x2x2 experimental design for A) total phenolic compounds, B) total flavonoid compounds, C) extraction yield and D) DPPH radical scavenging activity. Notes: OfiM, *O. ficus-indica* Milpa Alta; Oe, *O. engelmannii*; OfiC, *O. ficus-indica* Copena; MeOH, methanol; EtOH, ethanol.

As seen in **table I**, 1:10 solid-liquid ratio is widely applied in literature for extractions, as well as methanol and ethanol at 80%, being the best solvents for polyphenolic compound extraction. The best results for TPC (8.99 mg GAE/g) in our work were in the range reported by Aruwa et al. (2019a) and were obtained in a shorter extraction period than most of the literature. An improvement of the extraction time would be by applying ultrasound extraction (Brahmi et al., 2022).

**Biomethane potential assays**

In the first set of experiments (**Table II**), the best maximum biomethane yield ($B_0$) was OfiM at ISR 2.5 (552 mL $CH_4$/g VS), followed by 20% less in Oe at 2.5 ISR (432 mL $CH_4$/g VS). It can be observed that $B_0$ increases as ISR increases in the assayed range, as well as pH; at low ISR, pH was the lowest. Then, lower biomethane yields at 0.5 ISR could be because of inhibition due to high solids concentration that led to the accumulation of VFAs (**Table II**). Indeed, the volatile solids removed (VSrem) were higher while ISR decreased, as well as alkalinity and VFAs final concentration. Thus, buffering capacity as alkalinity was constrained leading to a slight acidification.

Regarding the rate constant (k), for OfiM it rose as ISR diminished, whereas for Oe was the opposite. However, as **figure 2** shows, the cumulative biomethane yield behavior of OfiM at ISR 2.5 was notably better than others.

Since raw *Opuntia* biomass digestion at ISR 2.5 resulted in the highest biomethane yield for either substrate, we proceeded to compare the raw biomasses versus solid extraction residues from the three types of *Opuntia* at ISR 2.5. **Figure 3** and **table III** show that raw biomasses gave the highest maximum biomethane potential. On average, biomethane yield was reduced by approximately 6.7% when using extraction solid residues. This difference can be assumed to the fact that a fraction of the easily digestible sugars, such as pectin or mucilage (Kumar et al. 2008), is lost in the previous extraction process. As hydrocolloids are not soluble in methanol or ethanol, 20% water in the extraction step did interact with the biomass and could have recovered a fraction of the mucilage (Procacci et al. 2021). Interestingly, this experiment

**TABLE I.** Phenolic compounds extraction from *Opuntia.*

| Biomass | Extraction conditions | Results | Reference |
|---|---|---|---|
| *O. ficus-indica* cladodes | S:L 1:10<br>Methanol and ethanol 80% for 12 h | TPC: 7.23 – 9.86 mg GAE/g | Aruwa et al. (2019a) |
| *O. ficus-indica* cladodes | S:L 1:10<br>Successive extraction (methanol 50%, acetone 70% and water) 4 h at 25 °C | TPC: 39.26 mg GAE/g<br>TFC: 4.83 mg rutin/g | De Santiago et al. (2021) |
| *O. joconostle* fruit | Microwave assisted extraction<br>Water at 5 °C for 10 min | TPC: 9 – 13 mg GAE/g<br>TFC: 2.6 – 5.6 mg CE/g | Dávila-Hernández et al. (2019) |
| *O. ficus-indica* | S:L 1:10<br>Ethanol 70% at 25 °C for 5 d | TPC: 86 mg GAE/g<br>TFC: 4.8 mg QE/g | Dávila-Aviña et al. (2019) |
| *O. ficus-indica* Milpa Alta and Copena | S:L 1:10<br>Methanol and ethanol 80% at 25 °C for 1 h. | TPC: 2.66 – 8.99 mg GAE/g<br>TFC: 0.45 – 1.67 mg QE/g | This work |

Notes: S:L, solid-liquid ratio; TPC, total phenolic compounds; GAE, gallic acid equivalent; TFC, total flavonoid compounds; CE, catechin equivalents QE, quercetin equivalent.

**TABLE II.** First-order model kinetic parameters and digestate characterization for two types of *Opuntia* and three ISR biomethane potential assays.

| | Biomass | | | | | |
|---|---|---|---|---|---|---|
| | OfiM | | | Oe | | |
| **Parameters** | **2.5** | **1.5** | **0.5** | **2.5** | **1.5** | **0.5** |
| $B_0$ (mL $CH_4$/g VS) | 552 | 338 | 111 | 432 | 371 | 143 |
| $k$ ($d^{-1}$) | 0.25 | 0.39 | 1.12 | 0.25 | 0.2 | 0.12 |
| TS (% wb) | 13.43 | 13.6 | 18.52 | 14.66 | 15.41 | 19.24 |
| VS (% db) | 33.48 | 36.29 | 43.90 | 32.06 | 34.79 | 41.97 |
| VSrem (% db) | 24.41 | 30.15 | 33.23 | 20.98 | 23.84 | 33.07 |
| Alk (mg $CaCO_3$/L) | 8235 | 9475 | 16130 | 9300 | 8500 | 8750 |
| VFA (mg $CH_3COOH$/L) | 543 | 1185 | 5550 | 954 | 552 | 1302 |
| pH | 8.09 | 7.77 | 6.91 | 8.11 | 8.04 | 7.36 |

Notes: OfiM, *O. ficus-indica* Milpa Alta; Oe, *O. engelmannii*; $B_0$, maximum biomethane yield; $k$, reaction rate constant; TS, total solids; VS, volatile solids; VSrem, volatile solids removed; Alk, alkalinity; VGA, volatile fatty acids; wb, wet basis; db, dry basis.

gave lower results the previous one. Still, the values are in the high range for biomethane yields.

As for the other parameters evaluated, comparing *Opuntia* for raw biomass and extraction residues individually, there was no significant difference between their values. For instance, pH, alkalinity and VFAs for raw biomass were around 7.86, 7990 mg/L and 710 mg/L, respectively; whereas, for extraction residues, they were 7.89, 7188 and 486 mg/L (**Table III**).

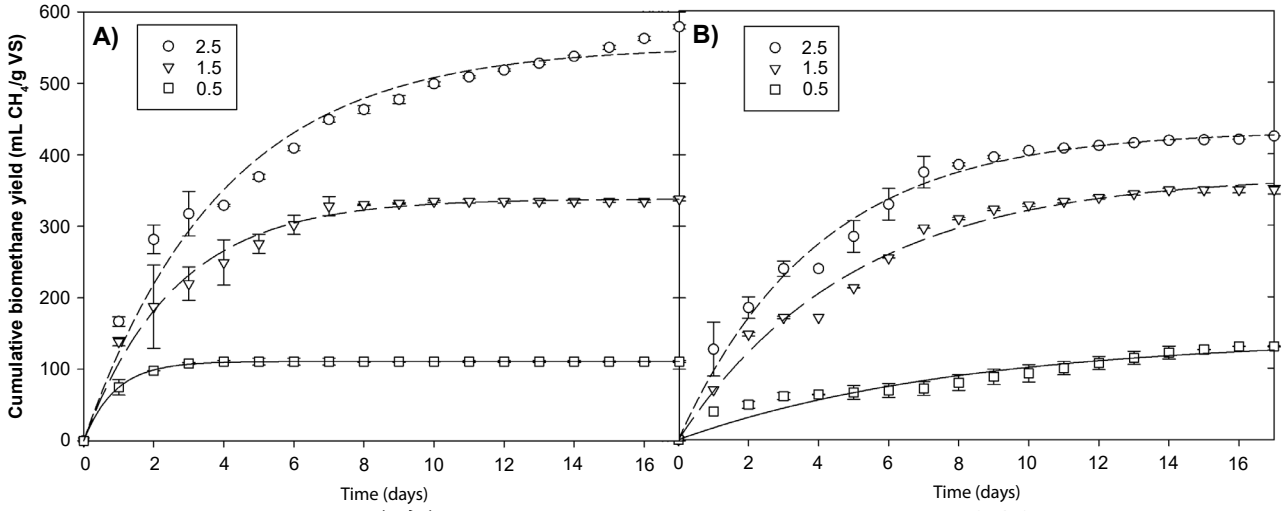


**Fig. 2.** Cumulative biomethane yield kinetics using A) OfiM and B) Oe as substrate at three ISR. Data adjusted to first-order model. Note: both graphs have the same scale.

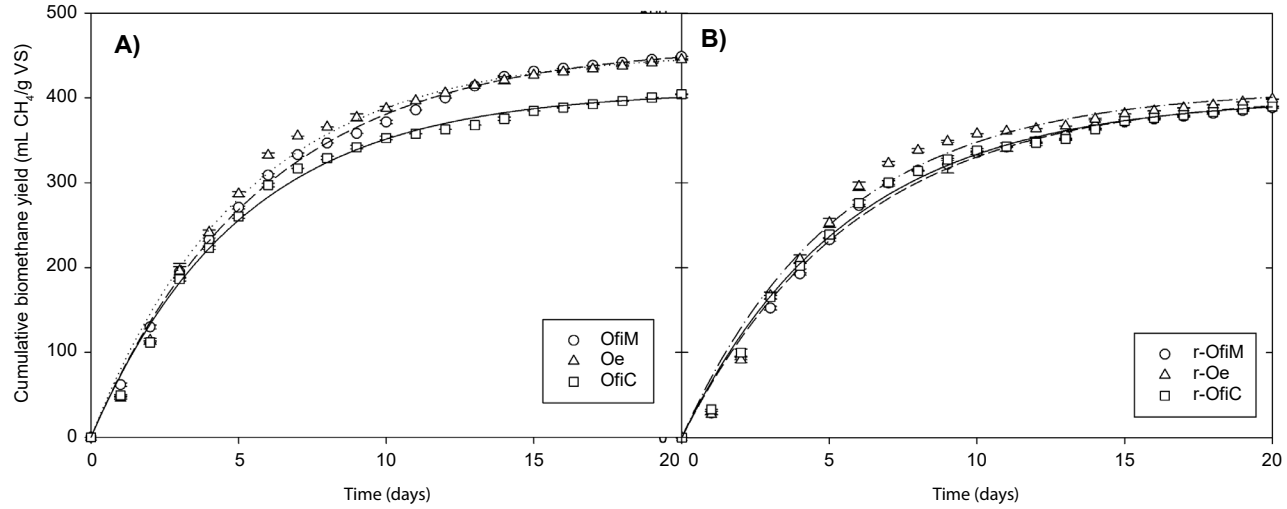


**Fig. 3.** Cumulative biomethane yield kinetics at ISR 2.5 for A) raw dry *Opuntia* cladodes and B) extraction solid residues. Note: both graphs have the same scale.

Since the differences between the type of biomass employed and maximum methane yield are minimal (ca. 7%), the extraction of phenolic compounds from *Opuntia* biomass and the subsequent treatment of its solid effluent by anaerobic digestion is feasible. An extract with antioxidant capacity and biomethane would be obtained, while valorizing effluents with reduced yield loss.

Finally, from the extraction residues of OfiC (r-OfiC) evaluation, we observe that the highest $B_0$ was 418.9 mL $CH_4$/g VS at ISR 0.5 (**Table IV**); nevertheless, this assay was the slowest biomethane producer as $k$ was 0.073 $d^{-1}$, meaning that such yield was achieved in a longer period as appreciated in **figure 4**. Since at 20 d the ISR 0.5 was 15% lower than the other assays, the ISR 2.5 was preferred as the best ratio due to a higher kinetic constant and no significative differences ($p > 0.364$, **Table SVI**). It is noteworthy that parameters analyzed for the three ISR were almost similar. Unlike raw OfiM and Oe biomethane potential assays, pH was constant at the three ISR evaluated; whereas alkalinity was moderated and balanced as well. This could explain why maximum biomethane yields appear average at this ISR

**TABLE III.** First-order model kinetic parameters and digestate characterization for raw biomass and extraction residues at ISR 2.5 biomethane potential assays.

| | Raw biomass | | | Extraction residues | | |
|---|---|---|---|---|---|---|
| **Parameters** | **OfiM** | **Oe** | **OfiC** | **r-OfiM** | **r-Oe** | **r-OfiC** |
| $B_0$ (mL $CH_4$/g VS) | 458.6 | 457.7 | 404.1 | 411.8 | 419 | 400.8 |
| k ($d^{-1}$) | 0.177 | 0.19 | 0.202 | 0.164 | 0.181 | 0.179 |
| TS (% wb) | 10.40 | 10.45 | 10.75 | 10.05 | 10.67 | 10.41 |
| VS (% db) | 31.73 | 31.66 | 32.4 | 32.07 | 32.14 | 31.67 |
| VSrem (% db) | 26.46 | 26.89 | 24.51 | 26.97 | 23.87 | 27.92 |
| Alk (mg $CaCO_3$/L) | 8240 | 7925 | 7805 | 7235 | 6980 | 7350 |
| VFA (mg $CH_3COOH$/L) | 774 | 648 | 708 | 426 | 342 | 690 |
| pH | 7.83 | 7.87 | 7.9 | 7.95 | 7.91 | 7.83 |

Notes: OfiM, *O. ficus-indica* Milpa Alta; Oe, *O. engelmannii*; OfiC, *O. ficus-indica* Copena; $B_0$, maximum biomethane yield; *k*, reaction rate constant; wb, wet basis; dry basis.

**TABLE IV.** First-order model kinetic parameters and digestate characterization for r-OfiC at three ISR biomethane potential assays.

| | r-OfiC | | |
|---|---|---|---|
| **Parameters** | **2.5** | **1.5** | **0.5** |
| $B_0$ (mL $CH_4$/g VS) | 400.8 | 408.9 | 418.9 |
| k ($d^{-1}$) | 0.179 | 0.168 | 0.073 |
| TS (% wb) | 10.41 | 11.29 | 13.10 |
| VS (% db) | 31.67 | 32.40 | 34.54 |
| VSrem (% db) | 27.92 | 31.69 | 49.65 |
| Alk (mg $CaCO_3$/L) | 7350 | 7325 | 7880 |
| VFA (mg $CH_3COOH$/L) | 690 | 834 | 1272 |
| pH | 7.83 | 7.91 | 7.90 |

Notes: OfiM, *O. ficus-indica* Milpa Alta; Oe, *O. engelmannii*; $B_0$, maximum biomethane yield; *k*, reaction rate constant; wb, wet basis; db, dry basis.

range (**Table IV**). Still, VSrem was different for each ISR, but kept the trend observed in previous experiments, where at lower ISR, the higher the VSrem. This was similar for VFA concentration.

*Opuntia* biomasses exceeded both the theoretical methane yield in glucose basis (373 mL $CH_4$/g glucose) and by the Boyle equation (Raposo et al. 2011) that considered the *Opuntia* empirical formulae as $C_{29}H_{60}O_{32}N$ (325 mL/g VS, Eq.4). Such higher yields could be attributed to the high metabolic activity of the inoculum or the consumption of a part of the inoculum, resulting in increased biomethane

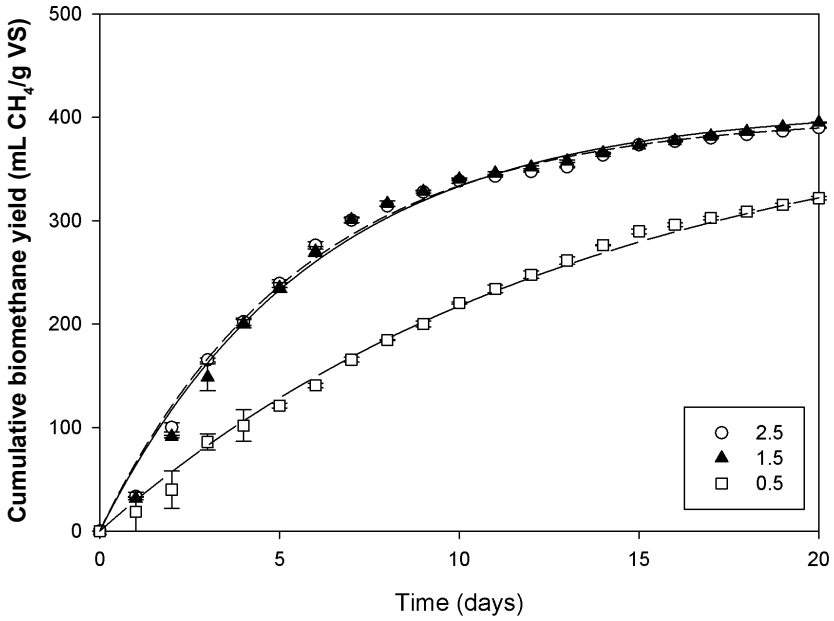


**Fig. 4.** Cumulative biomethane yield kinetics of r-OfiC at three ISR.

**TABLE V.** Maximum biomethane yields from *Opuntia*.

| Substrate | ISR | Yield (mL $CH_4$/g VS | References |
|---|---|---|---|
| *O. ficus-indica* | 3 | 430 | Calabrò et al., 2018 |
| *O. ficus-indica* hydrolysate | 3 | 604 | Calabrò et al., 2018 |
| *O. maxima* + *Scenedesmus* | 2 | 360 | Ramos-Suárez et al., 2014 |
| *O. ficus-indica* | 2 | 300 | Lueangwattanapong et al., 2020 |
| *O. ficus-indica* + Cow manure | NR | 152.7 | Espinosa-Solares et al., 2022 |
| *O. ficus-indica* var. Milpa Alta | 2.5 | 552 | This work |
| *O. engelmannii* | 2.5 | 432 | This work |

Notes: ISR, inoculum-to-substrate ratio; VS, volatile solids; NR, not reported.

production due to endogenous bacterial metabolism (Calabrò et al. 2018). These findings are noteworthy as the methane production by the control was already subtracted, indicating that *Opuntia* biomass may enhance such endogenous metabolism. Indeed, the biomethane yields are comparable to those of Calabrò et al. (2018), who reported 430 and 600 mL $CH_4$/g VS using *O. ficus-indica* and its acid hydrolysate, respectively, at ISR 3 (**Table V**). On the other hand, Ramos-Suarez et al. (2014) performed co-digestion of *Scenedesmus* and *O. maxima* in a 25:75 ratio at ISR 2, and also obtained a high yield of 360 mL $CH_4$/g VS. Our results were above this results and those of Rodríguez-Valderrama et al. (2020) who obtained 342 mL $CH_4$/g VS using engineered processing: the anaerobic digestion of solid hydrolysates of fruit and vegetable residues in co-digestion with corn stover at ISR 4. It is noteworthy that the mentioned authors

have obtained such high yields employing acid hydrolysis and co-digestion techniques that are useful for accessing and profiting biomass. However, there are some drawbacks in the procedure conditions, such as high temperatures, use of concentrated or diluted acid that can lead to corrosion and degradation of equipment, but most importantly, the formation of inhibitors such as furfural and hydroxymethylfurfural (Jędrzejczyk et al. 2019). Therefore, using *Opuntia* as a substrate for biofuel production is highly advisable, requiring more experimentation to correctly assess the potentialities of this extraordinary biomass.

## CONCLUSIONS

The best solvent for extracting antioxidant compounds for all varieties of *Opuntia* tested was 80% methanol. OfiM gave the best results for all responses analyzed: 8.99 mg GAE/g, 1.67 mg QE/g, and 58.5% DPPH scavenging, all within the range of the literature and obtained in a shorter extraction period.

The maximum yield for anaerobic digestion of OfiM and Oe was 552 and 432 mL $CH_4$/g VS, respectively, at ISR 2.5. The results led to infer that *Opuntia* biomass may enhance endogenous metabolism that allows yields above the theoretical from the SV of the substrate.

The incorporation of the antioxidant extraction stage before the anaerobic digestion of *Opuntia* was achieved with a 7% decrease in methane production. Still, this two-step process must add future improvements on residue utilization, and there is need to evaluate the environmental impact of the election of the processes shown here and other further configurations.

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# SUPPLEMENTARY MATERIAL

**TABLE SI.** Experimental design for the evaluation of *Opuntia* species, solvent and concentration.

| Opuntia | Solvent | Concentration % | TPC mg GAE/g | TFC mg QE/g | Yield g/L | DPPH % scavenging | TSg g/L | RSg g/L |
|---|---|---|---|---|---|---|---|---|
| OfiM | MeOH | 100 | 3.68 | 0.49 | 5.89 | 18.00 | 0.53 | 0.64 |
| | EtOH | | 1.15 | 0.22 | 1.44 | 5.68 | 0.14 | 0.13 |
| | MeOH | 80 | 8.99 | 1.67 | 20.49 | 58.52 | 2.06 | 1.37 |
| | EtOH | | 7.49 | 1.59 | 14.29 | 47.69 | 2.00 | 1.45 |
| Oe | MeOH | 100 | 1.32 | 0.27 | 10.18 | 22.09 | 1.08 | 0.57 |
| | EtOH | | 0.66 | 0.11 | 2.49 | 1.64 | 0.25 | 0.39 |
| | MeOH | 80 | 2.66 | 0.45 | 16.41 | 52.12 | 2.71 | 0.84 |
| | EtOH | | 2.66 | 0.46 | 11.78 | 45.14 | 2.77 | 0.86 |
| OfiC | MeOH | 100 | 0.74 | 0.64 | 4.78 | 28.83 | 0.98 | 1.27 |
| | EtOH | | 0.44 | 0.52 | 1.36 | 19.39 | 0.22 | 0.45 |
| | MeOH | 80 | 6.25 | 0.67 | 14.11 | 39.22 | 3.49 | 2.92 |
| | EtOH | | 4.76 | 0.83 | 11.30 | 34.41 | 3.31 | 3.33 |

Notes: MeOH, methanol; EtOH, ethanol; TPC, total phenolic compounds; TFC, total flavonoid compounds; TSg, total sugars; RSg, reducing sugars.

**TABLE SII.** General factorial design ANOVA for total phenolic compounds.

| Source of variation | Sum of squares | Degrees of freedom | Mean squares | F | p value |
|---|---|---|---|---|---|
| Model | 183.49 | 11 | 16.68 | 143.95 | < 0.0001 |
| A-Opuntia | 50.50 | 2 | 25.25 | 217.91 | < 0.0001 |
| B-Solvent | 7.01 | 1 | 7.01 | 60.48 | < 0.0001 |
| C-Concentration | 102.49 | 1 | 102.49 | 884.52 | < 0.0001 |
| AB | 2.93 | 2 | 1.47 | 12.66 | 0.0011 |
| AC | 19.11 | 2 | 9.55 | 82.45 | < 0.0001 |
| BC | 0.04 | 1 | 0.04 | 0.37 | 0.5549 |
| ABC | 1.40 | 2 | 0.70 | 6.04 | 0.0153 |
| Error | 1.39 | 12 | 0.12 | | |
| Total | 184.88 | 23 | | | |

**TABLE SIII.** General factorial design ANOVA for total flavonoid compounds.

| Source of variation | Sum of squares | Degrees of freedom | Mean squares | F | p value |
|---|---|---|---|---|---|
| Model | 5.43 | 11 | 0.49 | 244.43 | < 0.0001 |
| A-Opuntia | 1.82 | 2 | 0.91 | 449.91 | < 0.0001 |
| B-Solvent | 0.04 | 1 | 0.04 | 18.51 | 0.0010 |
| C-Concentration | 1.95 | 1 | 1.05 | 964.17 | < 0.0001 |
| AB | 0.04 | 2 | 0.02 | 9.57 | 0.0033 |
| AC | 1.52 | 2 | 0.76 | 375.37 | < 0.0001 |
| BC | 0.07 | 1 | 0.07 | 35.01 | < 0.0001 |
| ABC | 0.00 | 2 | 0.00 | 0.66 | 0.5364 |
| Error | 0.02 | 12 | 0.00 | | |
| Total | 5.45 | 23 | | | |

**TABLE SIV.** General factorial design ANOVA for extraction yield..

| Source of variation | Sum of squares | Degrees of freedom | Mean squares | F | p value |
|---|---|---|---|---|---|
| Model | 874.65 | 11 | 79.51 | 345.09 | < 0.0001 |
| A-Opuntia | 33.23 | 2 | 16.61 | 72.10 | < 0.0001 |
| B-Solvent | 142.11 | 1 | 142.11 | 616.74 | < 0.0001 |
| C-Concentration | 645.84 | 1 | 645.84 | 2802.94 | < 0.0001 |
| AB | 9.93 | 2 | 4.97 | 21.55 | 0.0001 |
| AC | 37.18 | 2 | 18.59 | 80.69 | < 0.0001 |
| BC | 0.60 | 1 | 0.60 | 2.61 | 0.1321 |
| ABC | 5.76 | 2 | 2.88 | 12.50 | 0.0012 |
| Error | 2.76 | 12 | 0.23 | | |
| Total | 877.42 | 23 | | | |

**TABLE SV.** General factorial design ANOVA for extraction yield..

| Source of variation | Sum of squares | Degrees of freedom | Mean squares | F | p value |
|---|---|---|---|---|---|
| Model | 2230.95 | 11 | 202.81 | 132.02 | < 0.0001 |
| A-Opuntia | 7.36 | 2 | 3.68 | 2.40 | 0.1332 |
| B-Solvent | 213.90 | 1 | 213.90 | 139.24 | < 0.0001 |
| C-Concentration | 1676.43 | 1 | 1676.43 | 1091.28 | < 0.0001 |
| AB | 13.82 | 2 | 6.91 | 4.50 | 0.0349 |
| AC | 288.11 | 2 | 144.06 | 93.77 | < 0.0001 |
| BC | 19.53 | 1 | 19.53 | 12.71 | 0.0039 |
| ABC | 11.80 | 2 | 5.90 | 3.84 | 0.0514 |
| Error | 18.43 | 12 | 1.54 | | |
| Total | 2249.39 | 23 | | | |

**TABLE SVI.** One factor ANOVA for biomethane yield of r-OfiC.

| Source of variation | Sum of squares | Degrees of freedom | Mean squares | F | p value |
|---|---|---|---|---|---|
| Model | 4715 | 2 | 2357 | 1.444 | 0.3637 |
| A-ISR | 4715 | 2 | 2357 | 1.444 | 0.3637 |
| Pure Error | 4899 | 3 | 1633 | | |
| Cor Total | 9613 | 5 | | | |

**TABLE SVII.** Biomethane yields from OfiM and Oe species at three ISR.

| Opuntia | ISR | Q exp (mL) | Yexp sVS (mL/g sVS) | Yexp sVS (mL/g rVS) | Yexp sVS (mL/g remVS) |
|---|---|---|---|---|---|
| OfiM | 2.5 | 535.5 | 572.68 | 180.10 | 737.95 |
| | 1.5 | 562.0 | 366.64 | 159.05 | 527.46 |
| | 0.5 | 608.5 | 328.03 | 99.92 | 300.68 |
| Oe | 2.5 | 391.5 | 418.59 | 132.22 | 630.33 |
| | 1.5 | 584.3 | 382.19 | 166.03 | 696.27 |
| | 0.5 | 824.0 | 195.74 | 136.57 | 412.96 |

Notes: exp, experimental; Q, production; Y, experimental yield; sVS, substrate volatile solids; rVS, reactor volatile solids; remVS, removed volatile solids.

**TABLE SVIII.** Biomethane yields of raw and residual *Opuntia* species biomass.

| Opuntia | Q exp (mL) | Yexp sVS (mL/g sVS) | Yexp rVS (mL/g rVS) | Yexp remVS (mL/g remVS) ) |
|---|---|---|---|---|
| OfiM | 300.0 | 506.28 | 133.69 | 505.19 |
| Oe | 294.5 | 479.95 | 130.08 | 483.82 |
| OfiC | 269.0 | 408.'7 | 116.59 | 475.61 |
| r-OfiM | 2.5 | 471.72 | 118.36 | 438.91 |
| r-Oe | 1.5 | 443.67 | 118.52 | 496.49 |
| r-OfiC | 0.5 | 407.10 | 113.65 | 407.04 |

Notes: exp, experimental; Q, production; Y, experimental yield; sVS, substrate volatile solids; rVS, reactor volatile solids; remVS, removed volatile solids.

**TABLE SIX.** Biomethane yields of r-OfiC at three ISR.

| Opuntia | ISR | Q exp (mL) | Yexp sVS (mL/g sVS) | Yexp rVS (mL/g rVS) | Yexp remVS (mL/g remVS) ) |
|---|---|---|---|---|---|
| r-OfiC | 2.5 | 260 | 407.10 | 113.65 | 407.04 |
| | 1.5 | 433 | 412.18 | 161.69 | 510.22 |
| | 0.5 | 1032 | 348.88 | 230.07 | 464.20 |

Notes: exp, experimental; Q, production; Y, yield; sVS, substrate volatile solids; rVS, reactor volatile solids; remVS, removed volatile solids.