EFFICIENCY OF ETHANOL AND HEXANE EXTRACTION OF SUNFLOWER OIL FROM SEEDS OF PLANTS INOCULATED WITH AZOSPIRILLUM BRASILENSE AND FERTILIZED WITH SEWAGE SLUDGE

Abstract: Helianthus annuus L. is an annual oilseed crop that grows well in various types of soil. Sunflower oil can be used for several purposes, including human consumption, biodiesel production, and as an ingredient in cosmetics, pharmaceuticals, and veterinary products. Among some essential elements for plants is the use of nitrogen, which is one of the important nutrients for sunflower productivity. This study aimed to determine the efficiency of ethanol and hexane in extracting sunflower oil as well as assess the effects of sewage sludge application and inoculation with Azospirillum brasilense on sunflower oil yield. Sunflower oil was obtained from dried ground seeds by Soxhlet extraction. Thirty-eight samples were used and the experiment was carried out in a completely randomized block design. Each treatment had four repetitions. The following doses were applied: 0 (control); 5,000; 10,000; 20,000; and 40,000 kg/ha sewage sludge. Seeds were either inoculated (150 mL per 50 kg of seed) or not. Hexane and ethanol did not differ in extraction efficiency. The seeds inoculated with the bacteria and the presence of sewage sludge showed a higher yield, being 42% with higher doses of sewage sludge. The results show that ethanol, an environmentally friendly and sustainable solvent, is as efficient in extracting sunflower oil as hexane. Sludge fertilization and inoculation of sunflower with A. brasilense improve oil yield, probably by enhancing nitrogen fixation and uptake. The results show that ethanol, an environmentally friendly and sustainable solvent, is as efficient in extracting sunflower oil as hexane. Sludge fertilization and inoculation of sunflower with A. brasilense improve oil yield, probably by enhancing nitrogen fixation and uptake.

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Keywords: *Helianthus annuss* L., nitrogen, productivity, oil extraction

**Resumo:** *Helianthus annuss* L. é uma oleaginosa de ciclo anual, que apresenta crescimento rápido e se adapta a vários tipos de solo. O óleo de girassol pode ser utilizado para o consumo humano, produção de biodiesel e em indústrias de cosmética, farmacêutica, alimentícia, veterinária, entre outras. Dentre alguns elementos que são essenciais às plantas está o uso do nitrogênio, sendo este um dos importantes nutrientes para a produtividade do girassol. O presente trabalho teve por objetivo determinar a eficiência dos solventes hexano e etanol para a extração do óleo de girassol, bem como verificar a influência do lodo de esgoto e da inoculação da bactéria *Azospirillum brasilense* no rendimento do óleo de girassol. A extração por solvente do óleo de girassol foi realizada utilizando o aparelho *Soxhlet*, a partir das sementes secas e trituradas da planta. Foram utilizadas 38 amostras e o experimento foi conduzido em delineamento de blocos inteiramente casualizados. Cada tratamento teve quatro repetições. As seguintes doses foram aplicadas: 0; 5.000; 10.000; 20.000; e 40.000 kg/ha de lodo de esgoto. As sementes foram inoculadas (150 mL por 50 kg de semente) ou não. Os solventes hexano e etanol utilizados na extração não mostraram diferença significativa no rendimento do óleo de girassol. As sementes inoculadas com a bactéria e presença do lodo de esgoto apresentaram um maior rendimento, sendo de 42% com doses maiores de lodo de esgoto. Sendo assim, a utilização etanol para a extração do óleo de girassol torna-se viável e uma opção segura e sustentável, visto que o hexano é um solvente poluente e tóxico; além disso, a presença da *A. brasilense* e do lodo de esgoto nas sementes possivelmente proporcionaram um maior fornecimento de nitrogênio para a planta o que possibilitou melhor qualidade e, consequentemente, maior rendimento do óleo de girassol.

**Palavras-chave:** *Helianthus annuss* L., nitrogênio, produtividade, extração de óleo.
1. INTRODUCTION

Sunflower (*Helianthus annuus* L.) is a rustic oilseed that grows well in many types of soil. In addition, it is a species that has important characteristics such as resistance to drought, cold and heat, than most oilseeds grown in Brazil (CASTRO et al., 1996; FREITAS et al., 2012).

It is a plant that shows rapid maturation and short cycle, around 100 days. Such characteristics demonstrate that sunflower is an important option in agriculture in systems that involve crop rotation, as it has the capacity for nutrient cycling in the soil, which provides the development and improvement of the nutritional status of other crops (VALERIANO et al., 2020).

Sowing sunflowers generally occurs in August and September and their cultivation is mainly due to the presence of oil in the seeds, with a content of 36 to 47% (LOBO et al., 2012; SOARES et al., 2015; LEWANDOSKI et al., 2017). It is a species that has several applications, such as ornamental purposes, the use of grains for animal feed and the extraction of oil for human consumption, which is used in the cosmetic, pharmaceutical and veterinary industries; in addition to being used in the production of biodiesel (OLIVEIRA, 2004; NOBRE, 2012; ANDRIANASOLO et al., 2016; LEWANDOSKI et al., 2017).

Although oil yield may be related to plant genetic factors, other factors such as excess water, temperature, light and the presence of specific nutrients can also affect the quality and productivity of the plant (d'AVILA et al., 2016).

According to Schwerz et al. (2016), nitrogen is the most required nutrient for sunflower, after boron, and in addition to phosphorus and potassium. Nitrogen may become available to plants through biological fixation of atmospheric nitrogen. Diazotrophic microorganisms play a crucial role in nutrient cycling and nitrogen supply in both natural and managed ecosystems (MOREIRA et al., 2010). The element can also be supplied via organic soil amendments, such as
sewage sludge, which is rich in organic matter, phosphorus, and essential micronutrients, including iron and zinc (MELO et al., 2000).

In this context, vegetable oils are composed mostly of triglycerides (about 95%) and small quantities of mono- and diglycerides, free fatty acids, glycolipids, proteins, phospholipids, and sterols (WEISS, 1970; KRAUSE; MAHAN, 1991; MORETTO; FEET, 1998). When extracted for human consumption, vegetable oils must be refined for elimination of free fatty acids, proteins, natural dyes, moisture, and volatile and inorganic compounds (MORETTO; FEET, 1998). Different extraction methods can be used, such as mechanical pressing, solvent extraction, and supercritical fluid extraction. Soxhlet extraction with ethanol or hexane is an interesting technique that affords high oil yields in comparison with other methods. Oilseeds remain in contact with the solvent as it continuously evaporates and condenses (BRUM et al., 2009).

Hexane, a petroleum distillate, is the most common solvent for oil extraction from oilseeds. It is composed of a mixture of hexane isomers (boiling range, 65-71 °C), 45 to 70% of which is n-hexane. n-Hexane is considered a toxin in some countries, as it is highly neurotoxic at high concentrations (HAMMOND et al., 2005).

However, ethanol is an environmentally friendly alternative to hexane. It is safer, easier to handle, and less flammable than hexane, can be produced through biotechnological routes, and does not generate toxic waste. Furthermore, ethanol is widely available, reusable, and economically viable (SILVA et al., 2020). This study aimed to assess the efficiency of hexane and ethanol in extracting sunflower oil and determine whether inoculation with *A. brasilense* and fertilization with sewage sludge affects the yield of sunflower oil for biodiesel production.
2 THEORETICAL REFERENCE

2.1 Sunflower Crop

Nitrogen is the most limiting nutrient in sunflower (SCHWERZ et al., 2016). Chemical nitrogen fertilizers derived from non-renewable fossil fuels represent a major cost in sunflower production. Biological nitrogen fixers are an alternative to fossil fuel-derived fertilizers; nitrogen fixers may complement or even replace chemical fertilizers (REIS JÚNIOR et al., 1998; BERGAMASCHI, 2006; CARMO et al., 2020) at a low cost and with reduced environmental impacts, contributing greatly to agricultural sustainability (HUNGRIA et al., 2007).

Diazotrophs, also known as nitrogen-fixing prokaryotes, can be free-living, associative, or symbiotic. These microorganisms have been shown to contribute to nitrogen supply in natural and managed ecosystems (MOREIRA et al., 2010; CARMO et al., 2020). Diazotrophic bacteria of the genus Azospirillum are associative, that is, they do not form a symbiosis with the host plant (BASHAN; de-BASHAN, 2005). Associative bacteria excrete only part of the fixed nitrogen, not being able to completely meet the nitrogen requirements of plants (DOBBELAERE et al., 2003). In such cases, additional nitrogen sources must be supplied.

Sewage sludge is rich in nitrogen and organic matter and has adequate levels of phosphorus and essential micronutrients, such as iron and zinc (MELO; MARQUES, 2000; TSUTYA, 2001). Sludge fertilization is an interesting strategy to increase crop yield and promote sustainable development in agricultural systems (LOBO et al., 2012).

Organic matter is a major factor affecting the chemical, physical, and biological characteristics of soil (STEVENSON, 1994). Humic acids, major components of soil organic matter, can increase the population of diazotrophic bacteria and, consequently, enhance their beneficial effects on the host plant.
(MARQUES JÚNIOR, 2006). These effects are likely associated with the ability of humic acids to promote plant development and lateral and vertical root growth, increasing the number of colonization points for bacteria (CONCEIÇÃO et al., 2008).

2.2 Oil and Fat

Several seeds can be used for oil extraction, including sunflower, cotton, canola, soybean, castor, and peanut. Most vegetable oils are liquid at room temperature (ALMEIDA et al., 2013). Sunflower seed contains about 24% protein and 47% fatty material. Oleic and linoleic acids are the major fatty acids (90%). The quality of vegetable oils can be determined by their fatty acid composition. Triglycerides are formed by esterification of glycerol with three carboxylic acids (fatty acids) (BELTRÃO; OLIVEIRA, 2008), as shown in Figure 1.

Figure 1. Esterification of fatty acids. R * are the hydrocarbon chains of the fatty acids.

Fatty acids are composed of a carboxylic group (–COOH) attached to an alkyl chain of 4 to 36 carbon atoms. Fatty acid composition determines the melting point, viscosity, and suitability of oils as biodiesel. Fatty acids can be saturated (no double bonds), monounsaturated (one double bond), or polyunsaturated (two or more double bonds) (BELTRÃO; OLIVEIRA, 2008). Figure 2 - shows an example of two fatty acids with two double bonds between their carbon atoms.
Ricinoleic acid, the major component of castor oil, has 18 carbons, a hydroxyl group at C-12, and a double bond at C-9. Linoleic acid, an 18-carbon fatty acid with double bonds at C-9 and C-12, is a primary source of compounds that give flavor to fried foods and is present in large quantities in soybean and sunflower oils.

2.3 Process of extraction of vegetable oil by solvents

Mechanical or expeller pressing is the oldest and most widely known method of vegetable oil extraction. Currently, vegetable oils are produced by mechanical or solvent extraction (BAÜMLER et al., 2016; RABONATO et al., 2017). This study used solvent extraction to obtain sunflower oil because it is more efficient than mechanical pressing. Solvents achieve almost complete oil removal regardless of the initial oil content of seeds, generating a residue with less than 1% oil (MORETTO; FETT, 1986; OETTERER et al., 2006;).

Seeds were ground prior to extraction to increase the surface area and facilitate solvent penetration. In ground seeds, the oil phase is present as a thin layer surrounding seed particles, which can be recovered by simple dissolution, and also within intact cells, which can be removed by diffusion. Oilseed extraction processes typically consist of two steps. The first step involves the rapid dissolution of oil with solvent molecules. The rate of the second step depends on the diffusion of oil and solvent through the semipermeable plant cell wall. Thus,
oil extraction has a fast initial phase followed by a slower phase (MORETTO; FETT, 1986).

The oil/solvent solution is commonly referred to as miscella. It is known that the rate of extraction is greatly dependent on the conditions of the oil/miscella/solvent system. Factors that facilitate the diffusion process can increase the rate of extraction, such as small particle size, solvent temperature close to the boiling point, and adequate moisture content (BAÜMLER et al., 2016).

2.3.1 Solvent for extraction

An ideal solvent for oil extraction has the following properties: i) high oil solubility at low temperatures; ii) high selectivity for the desired product (in this case, triglycerides); iii) low chemical reactivity with product and equipment; iv) low viscosity and surface tension to promote good percolation and surface wetting; v) easy removal from bran and oil by low-energy methods; vi) water immiscibility; vii) low boiling point or range; viii) low heat of vaporization; and iv) low environmental impact (OETTERER et al., 2006).

Hexane is the solvent universally adopted in oil extraction industries. The solvent is a mixture of \( n \)-paraffinic petroleum fractions with variable composition and, therefore, does not have a boiling point but rather a boiling range (OETTERER et al., 2006). The widespread use of hexane as extraction solvent is due to its physical and chemical properties. It is completely non-polar, readily dissolves oils, has low latent heat of vaporization, and does not react with pipes or equipment. However, hexane has high flammability, explosiveness, and toxicity (OETTERER et al., 2006; SILVA et al., 2020).

However, despite the high extractive power of hexane, it is used as a solvent requires great care and raises great concerns, ranging from storage and handling, to proper disposal (SILVA et al., 2005; SILVA et al., 2020). In addition,
hexane can be emitted during the extraction process, which needs to recover the solvent, being able to react with other pollutants forming oxozium and photochemical oxidants (BAÜMLER et al., 2016).

In face with concerns about the environment, safety and health, alternatives have emerged that aim to replace hexane with other ecologically and economically viable solvents. In this context, ethanol is a sustainable alternative, which presents itself as a less aggressive solvent and comes from renewable raw materials (CORREIA et al., 2010; BAÜMLER et al., 2016; LONGO et al., 2020).

The ethanol has properties opposite to hexane, as it is a polar, biodegradable, non-toxic substance with the potential for extraction processes. In addition, it becomes an attractive solvent due to low cost and availability, as it is produced in Brazil on a large scale (CORREIA et al., 2010; SILVA et al., 2020).

In solvent extraction, the solvent penetrates the seed and dissolves the oil, albuminous compounds, and pigments. The solution is evaporated and concentrated at a low temperature, allowing solvent recovery. The mixture obtained from solvent evaporation is then re-dissolved in ethanol to separate various essential oil components. Extracts obtained by this process have a strong color, as non-volatile pigments are retained. On the other hand, extracts obtained by hydrodistillation have a light color. The success of the extraction process depends mainly on the efficiency, selectivity, and quality of the solvent used (GAUTO; ROSA, 2013).

3. MATERIALS AND METHODS

Sunflower BRS 122 seeds were supplied by the State University of Maringá, Umuarama, Brazil. The experiment was carried out in a completely randomized block design in a 5 × 2 factorial arrangement (5 sludge doses × 2 levels of inoculation with A. brasilense). Each treatment had four repetitions. The following doses were applied: 0 (control); 5,000; 10,000; 20,000; and 40,000.
kg/ha sewage sludge. Seeds were either inoculated (150 mL per 50 kg of seed) or not. Inoculation was performed 1 h before planting.

Sunflower seeds were subjected to solid-liquid extraction using a Soxhlet extractor. Prior to extraction, the seeds were oven-dried at 60 ± 5 °C for 1 h, stored in airtight plastic bags, and ground using a household mixer to increase extraction efficiency.

4. RESULTS AND DISCUSSIONS

No differences in oil yield were observed between samples extracted with hexane or ethanol (Table 1), indicating that ethanol can be used as an alternative to hexane to extract sunflower oil.

In the studies conducted by Correia et al. (2010) and Baümler et al. (2016), reported the optimization of the use of ethanol as a substitute for hexane, showing the efficiency and viability of this solvent in the extraction process of sunflower oil.

Although hexane has an apolar molecule and has great solvency power for oilseeds; ethanol provides environmental and economic advantages over hexane, since it is a non-toxic solvent, less risk to be handled, with low cost and which can be obtained through fermentative processes, being considered a renewable raw material (BAÜMLER et al., 2016).

Table 1 - Analysis of variance for oil yield in sunflower seeds obtained from plants grown with sewage sludge doses (0; 5,000; 10,000 and 15,000 kg/ha) and two inoculation conditions (with...
and without inoculation with inoculated *Azospirillum brasilense* via seeds at a dose of 150 mL per 50 kg) using two solvents for extraction (Ethanol and Hexane).

| Font Variation               | GL | Value of F |
|------------------------------|----|------------|
| Sludge                       | 4  | 0.693 ns   |
| Solvent                      | 1  | 0.383 ns   |
| Bacteria                     | 1  | 0.068 ns   |
| Sludge X Bacteria            | 4  | 0.366*     |
| Sludge X Solvent             | 4  | 0.545 ns   |
| Bacteria X Solvent           | 1  | 0.515 ns   |
| Sludge X Bacteria X Solvent  | 4  | 0.572 ns   |

*significant at 5% probability of error by the F test.
ns: not significant by the F test at the level of 5% probability of error.

**Source:** Author, 2020.

Inoculation with *A. brasilense* and sewage sludge fertilization did not influence oil yield. However, we observed significant interaction effects between inoculation and sewage sludge dose (Table 2).

The oil yield of plants treated with dose 4 was higher when seeds were inoculated with *A. brasilense*. In contrast, for plants treated with dose 5, uninoculated seeds produced higher oil yields. This result may be associated with the presence of excess nitrogen, since its deficiency causes nutritional disorders, while its excess can affect grain production and cause a decrease in oil yield (VASCONCELOS et al., 2015; SCHWERZ et al., 2016).

In the studies conducted by Lobo e Grassi Filho (2007), in which they used sewage sludge as a nitrogen source for sunflower, found that increasing the sewage sludge doses did not obtain significant differences between treatments for oil yield.

In addition, the difference showed in table 2 may be associated with the amount of micronutrients, and the availability of ions can interfere with some nitrogen-fixing bacteria. According to Marschner (1986), biological nitrogen fixation can be compromised if plants lack molybdenum, a constituent of nitrogenase and nitrate reductase, enzymes required for nitrogen assimilation.
Table 2 - Oil yield (%) in sunflower seeds cut from plants grown with sludge doses (0; 5,000; 10,000 and 15,000 kg/ha) and two inoculation conditions (with and without inoculation with *Azospirillum brasilense* inoculated via seeds in the 150 mL dose per 50 kg).

| Sludge doses | Oil yield with bacteria | Oil yield without bacteria |
|--------------|-------------------------|---------------------------|
| 1            | 41,45 a A               | 42,75 ab A                |
| 2            | 42,37 a A               | 40,82 ab A                |
| 3            | 41,16 a A               | 43,26 a A                |
| 4            | 42,71 a A               | 38,58 b B               |
| 5            | 39,40 a B               | 42,63 ab A               |

Averages followed by the same letter, uppercase in the row and lowercase in the column, do not differ by the Tukey Test at the level of 5% probability.

Source: Authors, 2020.

3. CONCLUSIONS

Sunflower oil yield did not vary with solvent, indicating that ethanol, an environmentally friendly and less toxic alternative to hexane, can be successfully used for oil extraction. Inoculation with *A. brasilense* did not influence oil yield; however, significant interaction effects between inoculation and sewage sludge dose were observed. Sewage sludge can be used to replace mineral nitrogen fertilizer, reducing the costs of sunflower production.

ACKNOWLEDGEMENTS
The authors thank State University of Maringá - Campus Umuarama (CAU/CCA) and Federal Institute of Paraná - Campus Umuarama for the support.

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Edição Especial - I SISU (Simpósio Interdisciplinar de Sustentabilidade)

Enviado em: 09/02/2020

Aceito em: 10/11/2020

Editores responsáveis: Otávio Akira Sakai e Diane Belusso