How to Cite:
Balderas-Hernández, V., Medina-Rivero, E., Barba-De la Rosa, A., & De León-Rodríguez, A. (2020). Agave salmiana syrup improves the production of recombinant human interleukin-2 in Escherichia coli. Revista Mexicana De Ingeniería Química, 20(1), 399-412. https://doi.org/10.24275/rmiq/Bio2004

Vol. 20, No. 1 (2021) 399-412
Revista Mexicana de Ingeniería Química

Agave salmiana syrup improves the production of recombinant human interleukin-2 in *Escherichia coli*

El jarabe de *Agave salmiana* mejora la producción de interleucina 2 humana recombinante en *Escherichia coli*

V.E. Balderas-Hernández+, E. Medina-Rivero+, A.P. Barba-De la Rosa, A. De León-Rodríguez*

[1] *División de Biología Molecular. Instituto Potosino de Investigación Científica y Tecnológica (IPICyT), Camino a la Presa de San José 2055 Lomas 4a. Sección C.P. 78216, San Luis Potosí, S.L.P., México.*

Received: January 19, 2020; Accepted: April 30, 2020

Abstract

The expression of heterologous proteins in *Escherichia coli* is strongly affected by the type of carbon source used. In this work, the expression of a synthetic codon-optimized gene of human interleukin-2 in *E. coli* BL21-SI, carrying plasmid pET12a-rhIL2 is presented. Glucose, fructose or Agave syrup from *Agave salmiana*, were used as carbon sources for production of recombinant human IL-2 (rhIL-2) in 1.5-L bioreactor aerobic cultures using mineral medium. Codon optimization of the native hIL-2 gene eliminated the presence of 35 rare codons for *E. coli*, and improved the codon usage up to 76% compared with the native gene sequence. Cultures using 10 g/L glucose showed the lowest production of rhIL-2, and in contrast, cultures using fructose improved the production of rhIL-2 1.9-times. The utilization of fructose from Agave syrup enhanced the rhIL-2 production 3.9-times in comparison with cultures using glucose, reaching 103.42±6.61 $mg_{IL\text{-}2}$/L. Also, the specific rhIL-2 production rate (5.52±0.33 $mg_{IL\text{-}2}/g_{DCW}{\cdot}h$) and the biomass production (3.09±0.04 g_{DCW}/L) using Agave syrup were the highest observed. These results indicate that Agave syrup is an effective carbon source that stimulates the production of rhIL-2 and biomass. This research shows the potential utilization of Agaves to generate alternative and valuable biotechnological products along with the alcoholic beverages.

Keywords: Codon optimization, fructose, NaCl inducer, recombinant protein, synthetic gene.

Resumen

La expresión de proteínas heterólogas en *Escherichia coli* es afectada fuertemente por el tipo de fuente de carbono utilizada. En este trabajo se presenta la expresión de un gen sintético con codones optimizados de la interleucina-2 humana en *E. coli* BL21-SI, que porta el plásmido pET12a-hIL2. Se emplearon glucosa, fructosa y jarabe de Agave de *Agave salmiana* como fuentes de carbono para la producción de IL-2 humana recombinante (rhIL-2) en cultivos aerobios en biorreactores de 1.5 L, utilizando medio mineral. La optimización de codones del gen nativo hIL-2 eliminó la presencia de 35 codones raros para *E. coli*, y mejoró la utilización de codones hasta en un 76% en comparación con la secuencia nativa del gen. Los cultivos con 10 g/L de glucosa mostraron la producción más baja de rhIL-2 y en contraste los cultivos que usaron fructosa la producción de rhIL-2 mejoró 1.9-veces. El uso de fructosa de jarabe de Agave incrementó hasta 3.9-veces la producción de rhIL-2 en comparación con los cultivos utilizando glucosa, alcanzando 103.42±6.61 $mg_{IL\text{-}2}$/L. Además, la velocidad específica de producción de rhIL-2 (5.52±0.33 $mg_{peso\ seco\ IL\text{-}2}/g{\cdot}h$) y la producción de biomasa (3.09±0.04 $g_{peso\ seco}$/L) usando jarabe de Agave fueron las más altas observadas. Estos resultados indican que el jarabe de Agave es una fuente de carbono eficiente para estimular la producción de rhIL-2 y biomasa. Este trabajo demuestra el uso potencial de los Agaves para generar productos biotecnológicos alternativos y valiosos además de la producción de bebidas alcohólicas.

Palabras clave: Optimización de codones, fructosa, inductor NaCl, proteína recombinante, gen sintético.

* *Corresponding author. E-mail*: aleonr@me.com, aleonr@ipicyt.edu.mx
Tel. +52 444 834 2000, Fax +52 444 834 2010
+ Both authors contributed equally.
https://doi.org/10.24275/rmiq/Bio2004
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

The genus Agave includes plants native to Mexico, Central America and southwest regions of the USA, while Mexico harbors 75% of the total Agave plants diversity (Delgado-Lemus *et al*., 2014). Agaves have a high ecological, cultural and economic value to Mexico; their fibers are used as a multipurpose manufacturing material, as well as in bioremediation applications (Hernandez-Botello *et al*., 2019), heavy metal removal (Alcázar-Medina *et al*., 2019, 2020) and biofuels production (García-Amador *et al*., 2019; Gómez-Guerrero *et al*., 2019). But more importantly Agaves are used for production of alcoholic beverages (Torres *et al*., 2015). Nearly 53 Agave species are utilized to produce alcoholic beverages such as, tequila (Iñiguez-Muñoz *et al*., 2019), pulque (fermentation of the fresh sap) (Escalante *et al*., 2016), comiteco (Lara-Hidalgo *et al*., 2019), aguamiel (Romero-López *et al*., 2020), and mezcal; a distilled spirit obtained from fermented Agave syrup (De León-Rodríguez *et al*., 2006; Torres *et al*., 2015). *A. mapisaga*, *A. atrovirens*, *A. Americana*, and *A. salmiana* are the main species used for alcoholic beverages production. The syrup (or must) is obtained by thermal hydrolysis from these plants which contains high levels of carbohydrates where fructans represent up to 60% of the total soluble carbohydrates (Mancilla-Margalli and López 2006; Solís-García *et al*., 2019). Fructans are composed of polymeric units of fructose, and minor amounts of glucose and sucrose, although distributional composition will vary depending of the specie, age, growth conditions of the plant and the hydrolysis process (Michel-Cuello *et al*., 2015). This non-expensive high content of fermentable sugars is an attractive carbon source to be used in a vast range of biotechnological applications (Singh *et al*., 2019), such as the production of prebiotics for the specific stimulation of probiotic bacteria growth (Althubiani *et al*., 2019; López and Urías-Silvas 2007), production of organic acids and biofuels (Davis *et al*., 2011; Mielenz *et al*., 2015; Núñez *et al*., 2011), production of important biotechnological enzymes (Oliveira *et al*., 2016), or production of recombinant proteins (Cui *et al*., 2011; Ueno *et al*., 2018).

In this sense, utilization of non-complex culture media, such as defined media made with salts and a non-expensive sugar carbon is preferred in order to economically produce proteins on a commercial scale (Ahmad *et al*., 2018). Besides, complex media such as Luria-Bertani Broth (LB) or Terrific Broth that are routinely used for protein production, but they might contain insufficient concentration of fermentable sugars. This might cause a quick loss of the balanced growth of *Escherichia coli* culture, that will result in compromise the growth rate and final biomass production (Sezonov *et al*., 2007). In contrast, *E. coli* efficiently consumes fructose in minimal media M9, and interestingly this monosaccharide improves the biomass yield by 40% and up to 70% and the production of recombinant protein by 50% in comparison with cultures using glucose (using same *E. coli* strain and same sugar concentration) as carbon source (Aristidou *et al*., 1999). Along with other advantages, such as short duplication time, consuming non-expensive sugars, simple growth conditions, ability to growth under different concentrations of oxygen (Valdez-Cruz *et al*., 2017), broad molecular tools for transformation and expression, and extensive knowledge of its metabolism and genetics, *E. coli* remains as the primary host to produce recombinant proteins (Rosano and Ceccarelli 2014). Among the vast array of recombinant proteins produced in *E. coli*, cytokines are a large family of small proteins with central therapeutic applications such as mediating and regulating immunity, inflammation and hematopoiesis, and immune cell proliferation and differentiation (Feldmann 2008; Ramani *et al*., 2015; Tayal and Kalra 2008). Recombinant human cytokines are used in therapeutics for the treatment of malignant human illnesses, especially those related to anticancer immune responses. Also, recombinant cytokines are broadly used in research and are key players for the production of monoclonal antibodies and the development of diagnostic tests (Dinarello 2007; Ramani *et al*., 2015). The recombinant human interleukin 2 (rhIL-2) was one of the first cytokines exploited for development of tumor immunotherapy (Choudhry *et al*., 2018). hIL-2 is a globular, glycosylated protein of 15.5 kDa, is produced by human lymphocytes that have been stimulated by mitogens or antigens. The mature protein secreted by T cells has 133 amino acids, it consists of 4 antiparallel, amphipathic α helices that form a quaternary structure indispensable for execution of hIL-2 function, is variably glycosylated, but this glycosylation is not necessary for its biological activity (Malek 2008). Since hIL-2 can induce proliferation and differentiation of T-cells without affecting its activity, rhIL-2 is one of the three FDA approved cytokine-based therapeutics for metastatic melanoma. Administration of rhIL-2 has induced

curative and durable regressions in patients with metastatic melanoma (Glitza *et al*., 2018; Rosenberg *et al*., 1994) or with renal cell cancer (Alva *et al*., 2016; Curti *et al*., 2017).

The aim of this work was to utilize fructose from *Agave salmiana* syrup as carbon source for the production of recombinant hIL-2 (rhIL-2) using a synthetic gen whose codon usage was optimized for its expression in the bacterial system *E. coli* BL21-SI.

2 Materials and methods

2.1 Synthetic gen, plasmids, strains and culture media

The synthetic hIL-2 gene (Entelechon, Regensburg, Germany) was cloned in pET12a vector expression (Novagen, Darmstadt, Germany) under the control of T7 promoter, using *Nde*I and *Bam*HI restriction sites. The resulting construction was confirmed by DNA sequencing. The *E. coli* DH5α strain used for routine cloning and plasmid screening was grown at 37 °C in LB medium (containing per liter: 10 g peptone, 5 g yeast extract, 5 g NaCl). The strain *E. coli* BL21-SI (GIBCO, Darmstadt, Germany) used for the cloning and expression of the recombinant protein, was grown in LBON medium (LB without NaCl, since NaCl is the inducer of T7 polymerase under the control of *proU* promoter in the BL21-SI strain) at 37 °C (Donahue and Bebee 1996). The LB and LBON media were supplemented with ampicillin 100 μg/mL.

Minimal medium was used for production cultivations in bioreactor, containing per liter: 10 g of fructose (from Agave syrup) or glucose or fructose (analytical grade), 1.0 g $MgSO_4$, 3.5 g KH_2PO_4, 3.5 g $(NH_4)_2HPO_4$, 40 μg thiamine, 100 mg ampicillin and 3 mL of trace elements stock (containing per liter: 1 g $CuCl_2 \cdot 2H_2O$, 27 g $FeCl_3$, 2 g $CoCl_2 \cdot 6H_2O$, 0.5 g H_3BO_3, 2 g $Na_2MoO_4 \cdot 2H_2O$, 1 g $CaCl_2 \cdot 2H_2O$, 2 g $ZnCl_2$, and 100 mL HCl). pH was buffered to 7.4 with NaOH 1M prior to sterilization (15 min at 121 °C). Bacterial inoculum was cultured in minimal medium supplemented with yeast extract 5 g/L. Agave syrup (must) was kindly provided by Mezcalera Ipiña (Ahualulco, San Luis Potosi, Mexico) and it was obtained by cooking the *Agave salmiana* pineapples in a stone oven (thermal hydrolysis) for 48 h and then the syrup was obtained by mechanical pressing. In the laboratory, Agave syrup was centrifuged at 7000 × g for 10 min and pasteurized at 65 °C prior to be used, also its content of glucose and fructose was characterized by HPLC. But only traces of glucose were detected.

2.2 Bioreactor cultivation

A 1.5 L total volume bioreactor (Applikon, The Netherlands) equipped with pH, oxygen and temperature controller was used for batch culture experiments. The agitation rate was 250 rpm. Dissolved oxygen (DO) was measured with a polarographic oxygen electrode (AppliSens, Applikon, The Netherlands). Mixtures of air, N_2 and O_2 were supplied at 0.5 vvm to maintain the DO at 9% (0.48 mg/L, at 37 ºC and 0.9 atm) during all cultivation period. The temperature was maintained at 37 ºC by a water-recirculating heat exchanger. The pH was measured with a potentiometric sensor AppliSens (Applikon, The Netherlands). The pH of the medium was controlled at pH 7 by the automatic addition of 2 M NaOH. 1 L of minimal medium was inoculated to obtain an initial optical density at 620nm (OD_{620}) of 0.2. The expression of rhIL-2 was induced with 0.3 M NaCl when the OD_{620nm} was 0.6, around 3.5 h after post-inoculation. Samples were periodically taken to measure protein and total sugars concentration, and rhIL-2 production. Batch cultures were performed by triplicate using different lot of agave syrup.

2.3 Analytical methods

Total protein was measured by Lowry method (Lowry *et al*., 1951) at 590 nm using a UV-visible spectrophotometer (Varian Cary BIO-50, Palo Alto, CA), bovine serum albumin (BioRad, Hercules, CA) was used as standard. Concentration of glucose and fructose were measured by high-performance liquid chromatography system (Waters 600, Milford, MA, USA) equipped with a Shodex SP0810 column (Shodex, Jing An, Shanghai) maintained at 80 °C, and water (milliQ grade) as mobile phase at flow of 1 mL/min, coupled with a high sensitivity evaporative light scattering detector (Eurosep Instruments DDL 31, Cergy - Pontoise Cedex, France). Biomass was measured as optical density at 620nm (OD_{620}) using a UV-visible spectrophotometer (Varian Cary BIO-50, Palo Alto, CA), and then converted to dry cell weight, where one OD at 620 nm was equivalent to 0.37 g (dry cell weight, DCW)/L (Balderas-Hernandez *et al*., 2020).

Proteins were separated by 5-20% SDS polyacrylamide gradient gel using standard methods

with Tris-glycine buffer. Proteins were either stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA) or transferred to nitrocellulose paper for Western blot in Trans-blot semi dry electrophoretic transfer cell (Bio-Rad, Hercules, CA). The nitrocellulose paper (Amersham Biosciences, Piscataway, NJ) was blocked with 3% skimmed milk for 1 h. In order to detect rhIL-2, a polyclonal antibody anti-hIL2 (PeproTech, Rocky Hill, NJ, USA) was used at 0.2 μg/mL and goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Bio-Rad, Hercules, CA) diluted to 1:3000 was used as secondary antibody. The blot was developed with *p*-Nitro Blue Tetrazolium Chloride and 5-Bromo-4-Chloro-3-Indolyl Phosphate, *p*-toluidine salt (Amersham Biosciences, Buckinghamshire UK). The production of total rhIL-2 was quantified by densitometry analysis of nitrocellulose membranes using the Quantity One^{TM} v4.5 software (BioRad, Hercules, CA) and commercial rhIL-2 (Peprotech) as standard.

2.4 *Kinetic parameters calculation*

For the characterization of the strains used in this work, specific growth rate (μ), glucose or fructose consumption rate (q_S), and rhIL-2 production rate (q_{IL-2}), were determined. μ and q_S were calculated during exponential growth phase. Since growth rates and rhIL-2 production kinetics differed among studied conditions, q_{IL-2} was calculated considering only the rhIL-2 production phase, defined as the time period starting one sample (1 h) before rhIL-2 was detected up to the point when a sharp decrease in accumulation was observed. Bioreactor cultures were performed in triplicate, and the reported values represent the mean of the experiments performed.

3 Results

3.1 *Design of synthetic hIL-2 and codon optimization*

The nucleotide sequence of the coding region of the native hIL-2 was redesigned and optimized to improve the average codon usage for its expression in *E. coli*. The hIL-2 native sequence contained 35 codons with low usage frequency for *E. coli* ranged from 0.29 to 8.03% (using a 10% threshold and the Class II gene frequencies (Hénaut and Danchin 1996)), that accounted for the 26% of the entire gene sequence. Codon ACA was the most prevalent codon (n = 8) for threonine in the native sequence (Fig. 1), however it has the lower codon usage among the triplets that code for this amino acid.

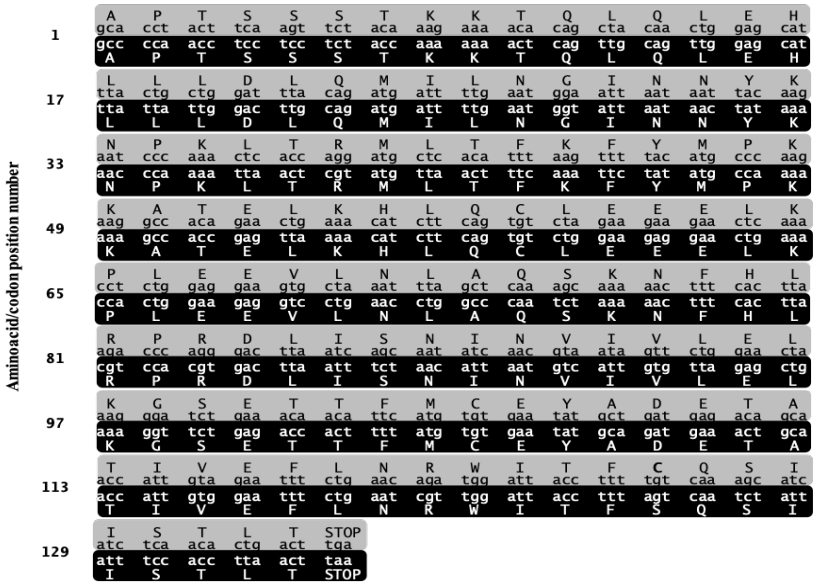

Fig. 1. Comparison of nucleotide and protein sequences of the native and optimized hIL-2. Grey sections correspond to the native hIL-2 codon sequence and its coding amino acid sequence. Black sections correspond to the optimized hIL-2 codon and its coding amino acid sequence.

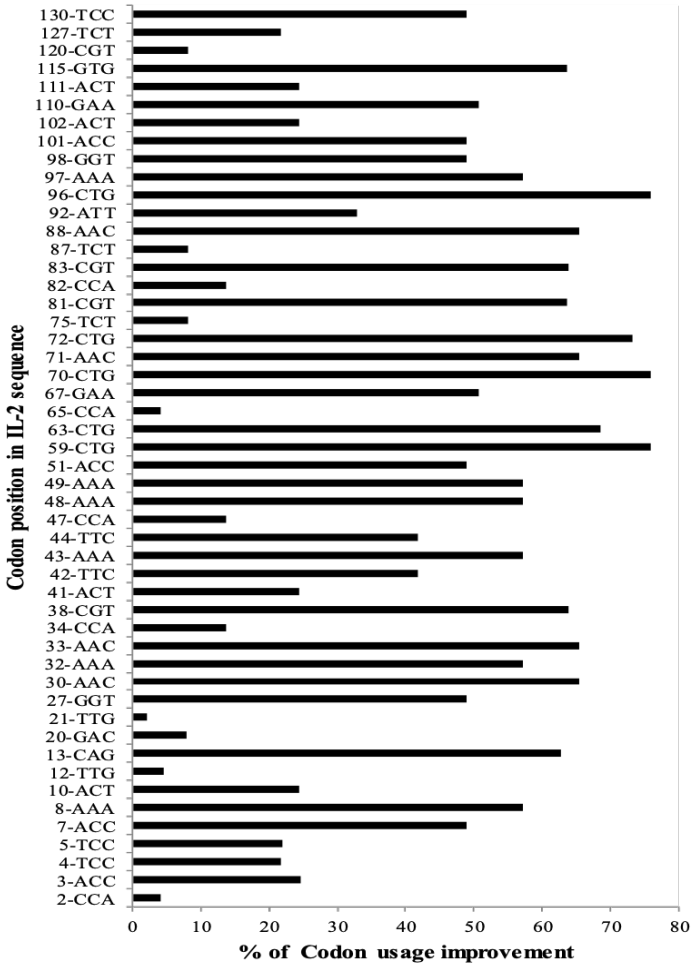

Fig. 2. Codon usage improvement in the optimized rhIL-2 gene. The % of improvement was considered using a 10% threshold and the Class II gene frequencies, (Hénaut and Danchin, 1996) in comparison with the native *hIL-2* gene codon sequence.

Thus, all ACA codons were replaced with codons of higher usage frequency in the optimized sequence. Codon usage in the synthetic *IL-2* gene sequence was improved in a range of 4 to 76% compared to the codons from the native sequence (Fig. 2). Triplets coding for leucine (L), asparagine (N), arginine (R), threonine (T) and lysine (K) were among the codons with the higher usage improvement up to 50% and above (Fig. 2). Comparison between synthetic and native IL-2 gene sequences showed a 39% of codon identity (Fig. 1).

3.2 rhIL-2 production using glucose

Bioreactor fermentations of *E. coli* BL21-SI/pET12a-rhIL2 strain for the production of rhIL-2 were evaluated using glucose, fructose or agave syrup, as carbon source. The culture of *E. coli* BL21-SI/pET12a-rhIL2 strain using glucose 10 g/L showed a specific growth rate (μ) of 0.18±0.01 h^{-1}, and reached a maximum final biomass of 1.33±0.05 g_{DCW}/L (Table 1) after 13.5 h of cultivation (Fig. 3). Glucose was completely consumed after 15.5 h of cultivation, and showed a specific glucose consumption rate (q_S) of 0.51±0.12 g/g_{DCW}·h, the highest of all observed q_S (Table 1). Although, *E. coli* BL21-SI/pET12a-rhIL2 strain showed a proficient growth on glucose, the production of the rhIL-2 protein was low, the lowest observed for all the evaluated sugars (Table 1), accumulating 26.2±1.15 $mg_{IL\text{-}2}$ /L at the end of the cultivation (Fig. 3).

Table 1. Kinetic parameters of fermenter cultures of *E. coli* BL21-SI/pET12a-rhIL2 for the production of recombinant hIL-2, using different carbon sources.

Carbon source	**Final biomass** (g_{DCW}/L)	μ (h^{-1})	q_S ($g/g_{DCW}\cdot h$)	q_{IL-2} ($mg_{IL\text{-}2}/g_{DCW}\cdot h$)	**Final rhIL-2** ($mg_{IL\text{-}2}$/L)
Fructose	1.16±0.22	0.12±0.02	0.45±0.05	4.30±0.05	50.17±8.17
Glucose	1.33±0.05	0.18±0.01	0.51±0.12	2.46±0.11	26.2±1.15
Fructose from Agave syrup	3.09±0.04	0.23±0.02	0.29±0.03	5.52±0.33	103.42±6.61

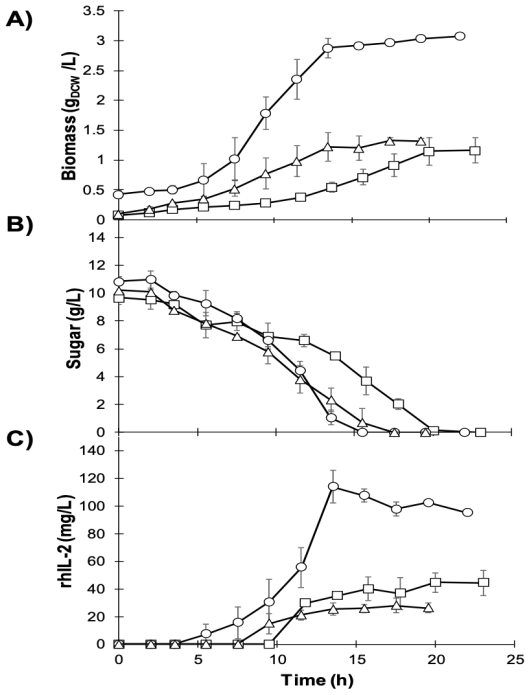

Fig. 3. Batch cultures in 1.5-L bioreactors of *E. coli* BL21-SI/pET12a-rhIL2 for the production of recombinant hIL-2. (A) Growth curves, (B) glucose or fructose consumption, and (C) recombinant hIL-2 production. Symbols: open circle (◯), cultures using fructose from Agave syrup; open square (□), cultures using fructose; open triangle (△), cultures using glucose. Graphs show results from the mean of triplicate experiments.

3.3 rhIL-2 production using fructose or agave syrup fructose

Concerning to the utilization of fructose (analytical grade) as carbon source for the production of rhIL-2. Strain *E. coli* BL21-SI/pET12a-rhIL2 showed a specific growth rate of 0.12±0.01 h^{-1} (Table 1), 33.4% lower than the observed for the cultures with glucose. This slow growth was reflected in a lower biomass accumulation of 1.16±0.22 g_{DCW}/L after 20 h of cultivation (Fig. 3), accompanied of slower q_S of 0.45±0.05 $g/g_{DCW}\cdot h$ (Table 1). Interestingly, strain *E. coli* BL21-SI/pET12a-rhIL2 produced 50.17±8.17 $mg_{IL\text{-}2}$ /L, with a specific rhIL-2 production rate (q_{IL-2}) of 4.30±0.05 $mg_{IL\text{-}2}/g_{DCW}\cdot h$, when fructose was used as carbon source (Fig. 3). Those values, were 1.9- and 1.7-times higher than the obtained for cultures using glucose, respectively.

For a final set of bioreactor cultures of *E. coli* BL21-SI/pET12a-hIL2 strain, the production medium was supplemented with 10 g/L of fructose from Agave syrup. As observed in figure 3A, a final biomass of 3.09±0.04 g_{DCW} /L was reached in 13 h of cultivation, this biomass accumulation was 2.3- and 2.6-times higher than the obtained for the comparative cultures using glucose and fructose, respectively (Table 1). And as the biomass, the value of μ of 0.23±0.02 h^{-1} followed a similar increment pattern (Table 1). rhIL-2 reached a maximum production of 103.42±6.61 $mg_{IL\text{-}2}$ /L, with a q_{IL-2} of 5.52±0.33 $mg_{IL\text{-}2}/g_{DCW}\cdot h$, at 13.5 h of cultivation, when using fructose from Agave syrup (Fig. 3 and Table 1). These IL-2 production values were 3.9- and 2.0- times higher than the values obtained for the recombinant protein using glucose or fructose, respectively (Table 1). The SDS-PAGE analysis from the cultures using fructose from Agave syrup showed the presence of recombinant hIL-2 with a band signal at 15.3 kDa, that corresponds to the expected molecular weight of the recombinant protein (Fig. 4a). The intensity of the band increased with respect of the cultivation time, reaching its maximal between 13-16 h (Fig. 4a). The identity of the band signal for rhIL-2 was confirmed by western blot analysis. As observed in Figure 4b, recombinant hIL-2 was detected with a molecular weight of 15.3 kDa, observing a band signal at same level as the commercial rhIL-2 standard (Fig. 4b).

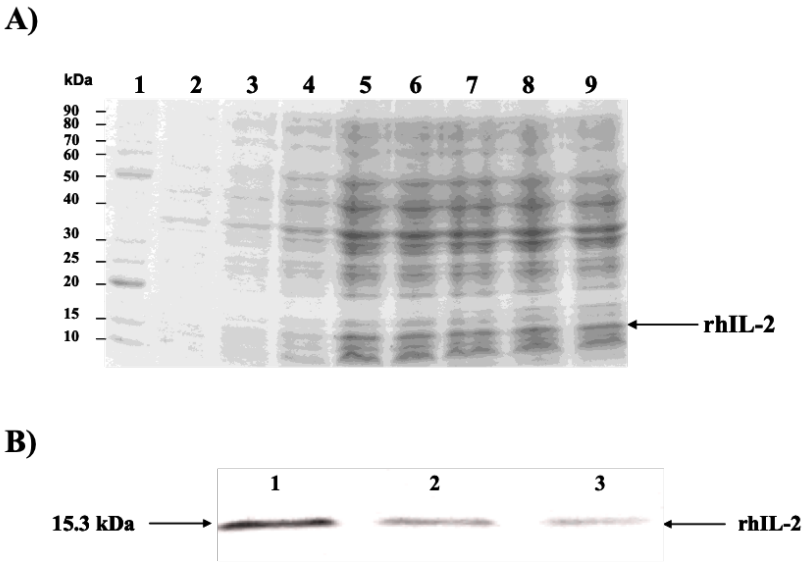

Fig. 4. Protein analysis of the production of rhIL-2 by strain *E. coli* BL21-SI/pET12a-rhIL2 using fructose from Agave syrup as carbon source. (A) SDS-PAGE electrophoretic protein pattern. Lane 1 shows protein molecular weight marker. Lanes 2-9 are 10 μL of total cells lysate at different time points of the culture, 3.5, 5.5, 7.5, 9.5, 11.5, 13.5, 15.5, 17.5, and 19.5, respectively. (B). Analysis of the expression of rhIL-2 by western blot. Lanes 1 and 2 are total cells lysate at 13.5 and 15.5 h of cultivation, respectively. Lane 3 is 50 ng of commercial rhIL-2 used as standard.

4 Discussion

E. coli remains as a model host for production of recombinant proteins, as around 30 to 40% of the approved therapeutic proteins are currently produced using this expression system (Baeshen *et al*., 2015; Walsh 2018). In order to surpass some of the drawbacks of using *E. coli* as system expression, the utilization of synthetic genes whose codon components have been optimized for its efficient transcription has improved (Elena *et al*., 2014). The codon-usage is a crucial factor that can influence the protein production efficiency through different mechanisms. It has been suggested that the translational elongation rate is influenced by codon bias, based on the correlation among the available levels of the different aminoacyl-tRNAs and their matching codons (Bulmer 1987; Ikemura 1985). Also, has been shown that genes encoding highly expressed proteins hold a strong bias toward codons with high frequency of usage in their sequence (Karlin *et al*., 2001). Besides, codon usage will affect the mRNA stability, protein secretion, protein folding and will impact on multiple aspects of mRNA/protein quality, quantity and function, and cellular physiology (Komar 2016). This indicates that genes whose codons have low frequency of usage will result in a lower amount of its correspondent expressed protein. As described, the native *hIL-2* gene contained in its sequence 26% of codons with low preference of usage for its expression on *E. coli*. In contrast, the optimized version of *hIL-2* gene contained codons with improved usage frequency (>50%), in order to avoid possible translation interrupts that potentially will affect its heterologous expression in *E. coli*. The multiple advantages of codon optimized genes have been demonstrated for the heterologous expression and efficient production of functional proteins at industrial scale (Elena *et al*., 2014; Gupta and Shukla 2016). Concerning to hIL-2, Williams *et al*., (1988) reported the expression of an optimized version of *hIL-2* gene, where the percentage of preferred codons was increased to 85% in comparison with the native sequence. This optimization increased the production up to 16 times more rhIL-2 than the native cDNA sequence when expressed in *E. coli* JM101. In another report, human *IL-2* gene was optimized to avoid rare codons and improve its efficient production using tobacco (*Nicotiana benthamiana*) as expression system (Matakas *et al*., 2013).

Strain *E. coli* BL21-SI/pET12a-rhIL2 reached the maximum production of the recombinant protein of 103.42±6.61 mg_{IL-2}/L, using fructose from Agave syrup as carbon source. In comparison with previous reports, rhIL-2 was expressed in *E. coli* BL21-SI/pEMR-hIL-2, reaching a final concentration of 200 mg_{IL-2}/L, in 20 h of cultivation (Medina-Rivero *et al*., 2007). High cell density cultures (around 100 OD_{680nm}) of *E. coli* HW21-2/pFC54.t, using glucose as carbon source, produced around 1830 mg_{IL-2} /mL (MacDonald and Neway 1990). Recombinant hIL-2 also has been expressed in other microorganisms, different from *E. coli*. Plasmid pPLGN1HIL2a, that contains human *IL-2* gene under the thermal inducible-pL promoter, was expressed in *Erwinia chrysanthemi, E. carotovora* and *Serratia marcescens*, producing 600, 525, and 780 U/mL of recombinant hIL-2, respectively. However, in contrast, the same plasmid expressed in *E. coli* MC1061 produced 4200 U/mL of recombinant hIL-2 (Leemans *et al*., 1987). In a comparative study, the fusion protein of hIL-2 and green fluorescent protein was expressed in *E. coli* BL21, yeast *Pichia pastoris* GS115, *Spodoptera frugiperda* Sf-9 insect cells, insect *Tricoplusia ni* larvae, and in *Drosophila melanogaster* S2 insect cells. The maximum total yield reported was 6.79, 1.70, 1.03, 22.74 and 2.55 mg_{IL-2}/L, respectively (Hyung *et al*., 2005).

Carbon source utilization by *E. coli* is an important factor to be considered since it has strong implications on its metabolism, cell growth, and ultimately on the production of the target recombinant protein (Hempfling and Mainzer, 1975). In most conditions, glucose is the preferred carbon source for *E. coli* since it provides faster growth and is consumed first in a mixture of sugars. As described, *E. coli* BL21-SI/pET12a-rhIL2 grew faster on glucose than with fructose (analytical grade), however the production of the rhIL-2 was the lowest observed. An explanation of this effect, might be that even when glucose utilization is related to high growth rates in *E. coli*, it may cause a high production of acetate even when oxygen is available (Szenk *et al*., 2017). This high accumulation of the conjugate base might cause osmostress perturbing the anion balance (Roe *et al*., 1998) of cells and affecting their metabolism, limiting its growth and its concomitant recombinant protein production, as extensively reported (Eiteman and Altman 2006; Lecina *et al*., 2013; Shi *et al*., 2017; Shiloach and Rinas 2009). In addition, it has been shown that *E. coli* is prone to a higher accumulation of acetate when is a host for recombinant protein overexpression, using glucose (Carneiro *et al*., 2013; Zeng and Yang 2019).

In contrast to the observed for cultures using glucose, the utilization of fructose or fructose from Agave syrup improved the expression of the IL-2 in *E. coli* BL21-SI/pET12a. A similar result was observed for the production of an exopolysaccharide (EPS) in *Scleroderma areolatum* Ehrenb, where the utilization of fructose as carbon source maximized the final EPS-yield and also its antiproliferative activity, in comparison to glucose-cultivations (Wu *et al*., 2019). Comparative cultivations of recombinant *E. coli* GJT001 overexpressing β-galactosidase showed that 40 mM of acetic acid was accumulated when glucose was supplemented. In contrast, no acetic acid was detected in cultures using defined media supplemented with fructose. This significant reduction in the wasteful acetate secretion resulted in an improved biomass yield, but more importantly a 65% recombinant β-galactosidase yield enhancement was obtained when equated to glucose cultivations (Aristidou *et al*., 1999). Du *et al*., (2016) reported that when strain *E. coli* K12f-pACLYC (*crtE, crtB* and *crtI*) containing the metabolic pathway for lycopene synthesis was grown in fructose, the final biomass accumulation and the final lycopene yield increased 3-fold and 7-fold than when growing on glucose, respectively. An explanation for this improved growth and product yield when fructose is used resides in the catabolic pathways of this hexose. Fructose utilization changed the gene expression patterns in comparison with glucose, enhancing the expression of genes related to the Embden-Meyerhof-Parnas pathway, the tricarboxylic acid cycle, and the oxidative phosphorylation. In addition, the expression of *ldhA, aceE* and *poxB*, which corresponding enzymes catalyze the production of lactate and acetate, were down-regulated by fructose (Du *et al*., 2016). These results are indicative that fructose is an optimal carbon source that promotes carbon flux through metabolic paths that will improve the availability of precursors, cofactors and energy carriers that support growth, and will decrease the accumulation of weak organic acids that lower the global performance of *E. coli*. Besides the observed benefits of fructose as carbon source, the utilization of Agave syrup as a source of fructose significantly improved the growth of *E. coli* BL21-SI/pET12a-hIL2, and more importantly the expression of recombinant hIL-2. This might be explained by the fact that besides fructose, Agave syrup contains other carbon skeletons, lipids, fatty acids and proteins, that might serve as

extra-carbon sources to improve growth of *E. coli* and it expression performance (Martínez-Aguilar and Peña-Álvarez 2009; Pinos-Rodríguez *et al*., 2008). Further investigation is needed to elucidate if short-chain fructooligosaccharides present in the Agave syrup might serve as extra carbon sources to improve production of IL-2 by *E. coli* BL21-SI/pET12a-rhIL2. As previously reported, 2% w/v of short chain β-fructans obtained from Salsify roots, improved the growth of *E. coli* PTCC 1330 in comparison with cultures using commercial inulin (Nourbakhsh *et al*., 2012). A similar improvement was observed for *E. coli*, under anaerobic conditions when 2% chicory inulin was supplemented into BSM-culture media, in contrast with control culture using glucose (López-Molina *et al*., 2005). Due to its complex composition further analyses, such as metabolite profiling using GC/MS and LC/MS, are recommended to describe better the composition of Agave syrup and the reproducibility during the production process. The results of present study indicate that the use of sugars from Agave syrup could be an alternative to other carbon sources for the production of rhIL-2 and others recombinant proteins of biotechnology interest. In the best of our knowledge, this is the first report focused in the utilization of Agave syrup for the production of a therapeutic recombinant protein.

Conclusions

Gene optimization of hIL-2 eliminates the presence of rare codons for its expression in *E. coli* BL21-SI and the utilization of Agave syrup as carbon source by *E. coli* BL21-SI/pET12a improves the production of biomass and the production of recombinant human IL-2, in comparison with cultures using glucose or fructose analytical grade. This research abilities the potential use the Agaves to produce alternative and valuable biotechnological products instead the alcoholic beverages.

Acknowledgements

E. Medina-Rivero thanks to CONACyT for PhD fellowship 157496.

References

Ahmad, I., Nawaz, N., Darwesh, N. M., ur Rahman, S., Mustafa, M. Z., Khan, S. B. and Patching, S. G. (2018). Overcoming challenges for amplified expression of recombinant proteins using *Escherichia coli*. *Protein Expression and Purification 144*, 12-18. https://doi.org/10.1016/J.PEP.2017.11.005.

Alcázar-Medina, F. A., Núñez-Núñez, C. M., Rodríguez-Rosales, M. D. J., Valle-Cervantes, S., Alarcón-Herrera, M. T. and Proal-Nájera, J. B. (2019). Lead removal from aqueous solution by spherical agglomeration using an extract of Agave lechuguilla Torr. as biosurfactant. *Revista Mexicana de Ingeniería Química 19*, 71-84. https://doi.org/10.24275/rmiq/Bio491.

Alcázar-Medina, F., Núñez-Núñez, C., Villanueva-Fierro, I., Antileo, C. and Proal-Nájera, J. (2020). Removal of heavy metals present in groundwater from a northern Mexico mining community using Agave tequilana Weber extracts. *Revista Mexicana de Ingeniería Química 19*, 1187-1199. https://doi.org/10.24275/rmiq/Bio1047.

Alva, A., Daniels, G. A., Wong, M. K. K., Kaufman, H. L., Morse, M. A., McDermott, D. F., Clark, J. I., Agarwala, S. S., Miletello, G., Logan, T. F., Hauke, R. J., Curti, B., Kirkwood, J. M., Gonzalez, R., Amin, A., Fishman, M., Agarwal, N., Lowder, J. N., Hua, H., Aung, S. and Dutcher, J. P. (2016). Contemporary experience with high-dose interleukin-2 therapy and impact on survival in patients with metastatic melanoma and metastatic renal cell carcinoma. *Cancer Immunology, Immunotherapy 65*, 1533-1544. https://doi.org/10.1007/s00262-016-1910-x.

Aristidou, A. A., San, K. Y. and Bennett, G. N. (1999). Improvement of biomass yield and recombinant gene expression in *Escherichia coli* by using fructose as the primary carbon source. *Biotechnology Progress 15*, 140-145. https://doi.org/10.1021/bp980115v.

Baeshen, M. N., Al-Hejin, A. M., Bora, R. S., Ahmed, M. M. M., Ramadan, H. A. I., Saini,

K. S., Baeshen, N. A. and Redwan, E. M. (2015). Production of biopharmaceuticals in *E. coli*: Current scenario and future perspectives. *Journal of Microbiology and Biotechnology 25*, 953-962. https://doi.org/10.4014/jmb.1412.12079.

Balderas-Hernandez, V. E., Landeros Maldonado, K. P., Sánchez, A., Smoliński, A. and De Leon Rodriguez, A. (2020). Improvement of hydrogen production by metabolic engineering of *Escherichia coli*: Modification on both the PTS system and central carbon metabolism. *International Journal of Hydrogen Energy 45*, 5687-5696. https://doi.org/10.1016/j.ijhydene.2019.01.162.

Bulmer, M. (1987). Coevolution of codon usage and transfer RNA abundance. *Nature 325*, 728-730. https://doi.org/10.1038/325728a0.

Carneiro, S., Ferreira, E. C. and Rocha, I. (2013). Metabolic responses to recombinant bioprocesses in *Escherichia coli*. *Journal of Biotechnology 164*, 396-408. https://doi.org/10.1016/j.jbiotec.2012.08.026.

Choudhry, H., Helmi, N., Abdulaal, W. H., Zeyadi, M., Zamzami, M. A., Wu, W., Mahmoud, M. M., Warsi, M. K., Rasool, M. and Jamal, M. S. (2018). Prospects of IL-2 in cancer immunotherapy. *BioMed Research International 2018*, 1-7. https://doi.org/10.1155/2018/9056173.

Cui, W., Wang, Q., Zhang, F., Zhang, S.-C., Chi, Z.-M. and Madzak, C. (2011). Direct conversion of inulin into single cell protein by the engineered Yarrowia lipolytica carrying inulinase gene. *Process Biochemistry 46*, 1442-1448. https://doi.org/10.1016/j.procbio.2011.03.017.

Curti, B., Daniels, G. A., McDermott, D. F., Clark, J. I., Kaufman, H. L., Logan, T. F., Singh, J., Kaur, M., Luna, T. L., Gregory, N., Morse, M. A., Wong, M. K. K. and Dutcher, J. P. (2017). Improved survival and tumor control with Interleukin-2 is associated with the development of immune-related adverse events: data from the PROCLAIMSM registry. *Journal for ImmunoTherapy of Cancer 5*, 102. https://doi.org/10.1186/s40425-017-0307-5.

Davis, S. C., Dohleman, F. G. and Long, S. P. (2011). The global potential for Agave as a biofuel feedstock. *GCB Bioenergy 3*, 68-78. https://doi.org/10.1111/j.1757-1707.2010.01077.x.

De León-Rodríguez, A., González-Hernández, L., Barba de la Rosa, A.P., Escalante-Mnakata, P., and López, M.G. (2006). Characterization of volatile compounds of mezcal, an ethnic alcoholic beverage obtained from *Agave salmiana*. *Journal of agricultural and food chemistry 54*, 1337-1341. https://doi.org/10.1021/jf052154+.

Delgado-Lemus, A., Casas, A. and Téllez, O. (2014). Distribution, abundance and traditional management of Agave potatorum in the Tehuacán Valley, Mexico: bases for sustainable use of non-timber forest products. *Journal of Ethnobiology and Ethnomedicine 10*, 63. https://doi.org/10.1186/1746-4269-10-63.

Dinarello, C. A. (2007). Historical insights into cytokines. *European Journal of Immunology 37*, S34-S45. https://doi.org/10.1002/eji.200737772.

Donahue, R. A. and Bebee, R. L. (1996). BL21-SI competent cells for protein expression in *E. coli*. Focus 21, 49-51. https://doi.org/10.1007/s10071-013-0651-x.

Du, W., Song, Y., Liu, M., Yang, H., Zhang, Y., Fan, Y., Luo, X., Li, Z., Wang, N., He, H., Zhou, H., Ma, W. and Zhang, T. (2016). Gene expression pattern analysis of a recombinant *Escherichia coli* strain possessing high growth and lycopene production capability when using fructose as carbon source. *Biotechnology Letters 38*, 1571-1577. https://doi.org/10.1007/s10529-016-2133-0.

Eiteman, M. A. and Altman, E. (2006). Overcoming acetate in *Escherichia coli* recombinant protein fermentations. *Trends in Biotechnology 24*, 530-536. https://doi.org/10.1016/j.tibtech.2006.09.001.

Elena, C., Ravasi, P., Castelli, M., Peiru, S. and Menzella, H. (2014). Expression of codon optimized genes in microbial systems: current industrial applications and perspectives.

Frontiers in Microbiology 5, 21. https://doi.org/10.3389/fmicb.2014.00021.

Escalante, A., López Soto, D. R., Velázquez Gutiérrez, J. E., Giles-Gómez, M., Bolívar, F. and López-Munguía, A. (2016). Pulque, a traditional Mexican alcoholic fermented beverage: historical, microbiological, and technical aspects. *Frontiers in Microbiology 7*, 1026. https://doi.org/10.3389/fmicb.2016.01026

Feldmann, M. (2008). Many cytokines are very useful therapeutic targets in disease. *The Journal of Clinical Investigation 118*, 3533-3536. https://doi.org/10.1172/JCI37346.

García-Amador, R., Hernández, S., Ortiz, I. and Cercado, B. (2019). Use of hydrolysate from Agave bagasse for bio-hydrogen production in microbial electrolysis cells. *Revista Mexicana de Ingeniería Química 18*, 865-874. https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n3/Garcia.

Glitza, I. C., Rohlfs, M., Guha-Thakurta, N., Bassett, R. L., Bernatchez, C., Diab, A., Woodman, S. E., Yee, C., Amaria, R. N., Patel, S. P., Tawbi, H., Wong, M., Hwu, W.-J., Hwu, P., Heimberger, A., McCutcheon, I. E., Papadopoulos, N. and Davies, M. A. (2018). Retrospective review of metastatic melanoma patients with leptomeningeal disease treated with intrathecal interleukin-2. *ESMO Open 3*, e000283. https://doi.org/10.1136/esmoopen-2017-000283.

Gómez-Guerrero, A. V, Valdez-Vazquez, I., Caballero-Caballero, M., Chiñas-Castillo, F., Alavéz-Ramírez, R. and Montes-Bernabé, J. L. (2019). Co-digestion of *Agave angustifolia* haw bagasse and vinasses for biogas production from mezcal industry. *Revista Mexicana de Ingeniería Química 18*, 1073-1083. https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n3/Gomez.

Gupta, S. K. and Shukla, P. (2016). Advanced technologies for improved expression of recombinant proteins in bacteria: perspectives and applications. *Critical Reviews in Biotechnology 36*, 1089-1098. https://doi.org/10.3109/07388551.2015.1084264.

Hempfling, W. P. and Mainzer, S. E. (1975). Effects of varying the carbon source limiting growth on yield and maintenance characteristics of *Escherichia coli* in continuous culture. *Journal of Bacteriology 123*, 1076-1087. https://doi.org/10.1128/JB.123.3.1076-1087.1975

Hénaut, A. and Danchin, A. (1996). Analysis and predictions from *Escherichia coli* sequences, or *E. coli in silico*. *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2047-2066. https://doi.org/10.1089/cmb.2014.0001.

Hernandez-Botello, M. T., Barriada-Pereira, J. L., Vicente, M. E. S. de, Mendoza-Pérez, J. A., Chanona-Pérez, J. J., López-Cortez, M. S. and Téllez-Medina, D. I. (2019). Determination of biosorption mechanism in biomass of Agave, using spectroscopic and microscopic techniques for the purification of contaminated water. *Revista Mexicana de Ingeniería Química 19*, 215-226. https://doi.org/10.24275/rmiq/IA501.

Hyung, J. C., Hwa, S. S., Hye, J. L., Hye, S. C., Dalal, N. N., Pham, M. Q. and Bentley, W. E. (2005). Comparative production of human interleukin-2 fused with green fluorescent protein in several recombinant expression systems. *Biochemical Engineering Journal 24*, 225-233. https://doi.org/10.1016/j.bej.2005.03.002.

Ikemura, T. (1985). Codon usage and tRNA content in unicellular and multicellular organisms. *Molecular Biology and Evolution 2*, 13-34. https://doi.org/10.1093/oxfordjournals.molbev.a040335.

Iñiguez-Muñoz, L. E., Arellano-Plaza, M., Oca, E. P.-M. de, Kirchmayr, M., Segura-García, L. E., Amaya-Delgado, L. and Gschaedler-Mathis, A. (2019). The production of esters and gene expression by *Saccharomyces cerevisiae* during fermentation on *Agave tequilana* juice in continuous cultures. *Revista Mexicana de Ingeniería Química 18*, 451-462. https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n2/Iniguez.

Karlin, S., Mrázek, J., Campbell, A. and Kaiser, D. (2001). Characterizations of highly expressed

genes of four fast-growing bacteria. *Journal of Bacteriology 183*, 5025- 5040. https://doi.org/10.1128/JB.183.17.5025-5040.2001.

Komar, A. A. (2016). The Yin and Yang of codon usage. *Human Molecular Genetics 25*, R77-R85. https://doi.org/10.1093/hmg/ddw207.

Lara-Hidalgo, C., Grajales-Lagunes, A., Ruiz-Cabrera, M. A., Ventura-Canseco, C., Gutiérrez-Miceli, F. A., Ruíz-Valdiviezo, V. M. and Archila, M. A. (2019). *Agave americana* honey fermentation by *Kluyveromyces marxianus* strain for "Comiteco" production, a spirit from mexican southeast. *Revista Mexicana de Ingeniería Química 16*, 451-462. https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n2/Iniguez

Lecina, M., Sarró, E., Casablancas, A., Gòdia, F. and Cairó, J. J. (2013). IPTG limitation avoids metabolic burden and acetic acid accumulation in induced fed-batch cultures of *Escherichia coli* M15 under glucose limiting conditions. *Biochemical Engineering Journal 70*, 78-83. https://doi.org/10.1016/j.bej.2012.10.006.

Leemans, R., Remaut, E. and Fiers, W. (1987). A broad-host-range expression vector based on the pL promoter of coliphage λ Regulated synthesis of human interleukin 2 in *Erwinia* and *Serratia* species. *Journal of Bacteriology 169*, 1899-1904. https://doi.org/10.1128/jb.169.5.1899-1904.1987.

López-Molina, D., Navarro-Martínez, M. D., Melgarejo, F. R., Hiner, A. N. P., Chazarra, S. and Rodríguez-López, J. N. (2005). Molecular properties and prebiotic effect of inulin obtained from artichoke (*Cynara scolymus* L.). *Phytochemistry 66*, 1476-1484. https://doi.org/10.1016/j.phytochem.2005.04.003.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Analytical Biochemistry 217*, 220-230. https://doi.org/10.1016/0304-3894(92)87011-4.

MacDonald, H. L. and Neway, J. O. (1990). Effects of medium quality on the expression of human interleukin-2 at high cell density in fermentor cultures of *Escherichia coli* K-12. *Applied and Environmental Microbiology 56*, 640-645. https://doi.org/10.1128/aem.56.3.640-645.1990.

Malek, T. R. (2008). The biology of interleukin-2. *Annual Review of Immunology 26*, 453-479. https://doi.org/10.1146/annurev.immunol.26.021607.090357.

Mancilla-Margalli, N. A. and López, M. G. (2006). Water-soluble carbohydrates and fructan structure patterns from Agave and Dasylirion species. *Journal of Agricultural and Food Chemistry 54*, 7832-7839. https://doi.org/10.1021/jf060354v.

Martínez-Aguilar, J. F. and Peña-Álvarez, A. (2009). Characterization of five typical Agave plants used to produce mezcal through their simple lipid composition analysis by gas chromatography. *Journal of Agricultural and Food Chemistry 57*, 1933-1939. https://doi.org/10.1021/jf802141d.

Matakas, J. D., Balan, V., Iv, W. F. C. and Gao, D. (2013). Plant-produced recombinant human interleukin-2 and its activity against splenic CD4+ T-cells. *International Journal of Life Sciences Biotechnology and Pharma Research 2*, 192-203.

Medina-Rivero, E., Balderas-Hernández, V. E., Ordoñez-Acevedo, L. G., Paz-Maldonado, L. M. T., Barba-De La Rosa, A. P. and De León-Rodríguez, A. (2007). Modified penicillin acylase signal peptide allows the periplasmic production of soluble human interferon-γ but not of soluble human interleukin-2 by the Tat pathway in *Escherichia coli*. *Biotechnology Letters 29*, 1369-1374. https://doi.org/10.1007/s10529-007-9395-5.

Michel-Cuello, C., Gallegos Fonseca, G., Maldonado Cervantes, E. and Aguilar Rivera, N. (2015). Effect of temperature and pH environment on the hydrolysis of maguey fructans to obtain fructose syrup. *Revista Mexicana de Ingeniería Química 14*, 615-622.

Mielenz, J. R., Rodriguez, M., Thompson, O. A., Yang, X. and Yin, H. (2015). Development of Agave as a dedicated biomass source:

production of biofuels from whole plants. *Biotechnology for Biofuels 8*, 79. https://doi.org/10.1186/s13068-015-0261-8.

Nourbakhsh, L., Sani, A. M., Mansoori, E. and Milani, E. (2012). Prebiotic effectiveness of β-fructan extracted from salsify on growth of *B. bifidum* and *E. coli*. *BioTechnology: An Indian Journal 6*, 341-346.

Núñez, H. M., Rodríguez, L. F. and Khanna, M. (2011). Agave for tequila and biofuels: An economic assessment and potential opportunities. *GCB Bioenergy 3*, 43-57. https://doi.org/10.1111/j.1757-1707.2010.01084.x.

Oliveira, L., Oliveira, T., Contiero, J. and Cazetta, M. (2016). Agave syrup as a substrate for inulinase production by *Kluyveromyces marxianus* NRRL Y-7571. Acta Scientiarum. *Biological Sciences 38*, 283. https://doi.org/10.4025/actascibiolsci.v38i3.31489.

Pinos-Rodríguez, J. M., Zamudio, M. and González, S. S. (2008). The effect of plant age on the chemical composition of fresh and ensiled *Agave salmiana* leaves. *South African Journal of Animal Sciences 38*, 43-50. https://doi.org/10.4314/sajas.v38i1.4108.

Ramani, T., Auletta, C. S., Weinstock, D., Mounho-Zamora, B., Ryan, P. C., Salcedo, T. W. and Bannish, G. (2015). Cytokines: The good, the bad, and the deadly. *International Journal of Toxicology 34*, 355-365. https://doi.org/10.1177/1091581815584918.

Roe, A. J., McLaggan, D., Davidson, I., O'Byrne, C. and Booth, I. R. (1998). Perturbation of anion balance during inhibition of growth of *Escherichia coli* by weak acids. *Journal of Bacteriology 180*, 767-772. https://doi.org/10.1128/JB.180.4.767-772.1998.

Romero-López, M. R., Osorio-Díaz, P., Flores-Morales, A., Robledo, N. and Mora-Escobedo, R. (2020). Chemical composition, antioxidant capacity and prebiotic effect of aguamiel (*Agave atrovirens*) during in vitro fermentation. *Revista Mexicana de Ingeniería Química 14*, 281-292. http://www.rmiq.org/ojs311/index.php/rmiq/article/view/1202

Rosano, G. L. and Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli*: advances and challenges. *Frontiers in Microbiology 5*, 172. https://www.frontiersin.org/article/10.3389/fmicb.2014.00172

Rosenberg, S. A., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H. and White, D. E. (1994). Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *JAMA 271*, 907-913. https://doi.org/10.1001/jama.1994.03510360033032.

Sezonov, G., Joseleau-Petit, D. and D'Ari, R. (2007). *Escherichia coli* physiology in Luria-Bertani broth. *Journal of Bacteriology 189*, 8746-8749. https://doi.org/10.1128/JB.01368-07.

Shi, X., Xie, J., Liao, S., Wu, T., Zhao, L.-G., Ding, G., Wang, Z. and Xiao, W. (2017). High-level expression of recombinant thermostable β-glucosidase in *Escherichia coli* by regulating acetic acid. *Bioresource Technology 241*, 795-801. https://doi.org/10.1016/j.biortech.2017.05.105.

Shiloach, J. and Rinas, U. (2009). Glucose and acetate metabolism in *E. coli* system level analysis and biotechnological applications in protein production processes. *Systems Biology and Biotechnology of Escherichia coli*, 377-400. https://doi.org/10.1007/978-1-4020-9394-4_18.

Singh, R. S., Singh, T. and Larroche, C. (2019). Biotechnological applications of inulin-rich feedstocks. *Bioresource Technology 273*, 641-653. https://doi.org/10.1016/j.biortech.2018.11.031.

Solís-García, A., Rivas-García, P., Escamilla-Alvarado, C., Rico-Martínez, R., Bravo-Sánchez, M. G. and Botello-Álvarez, J. E. (2019). Methanol production kinetics during agave cooking for mezcal industry. *Revista Mexicana de Ingeniería Química 16*, 827-834. http://rmiq.org/ojs311/index.php/rmiq/article/view/944

Szenk, M., Dill, K. A. and de Graff, A. M. R. (2017). Why do fast-growing bacteria enter overflow

metabolism? Testing the membrane real estate hypothesis. *Cell Systems 5*, 95-104. https://doi.org/10.1016/j.cels.2017.06.005.

Tayal, V. and Kalra, B. S. (2008). Cytokines and anti-cytokines as therapeutics-An update. *European Journal of Pharmacology 579*, 1-12. https://doi.org/10.1016/J.EJPHAR.2007.10.049.

Torres, I., Casas, A., Vega, E., Martínez-Ramos, M. and Delgado-Lemus, A. (2015). Population dynamics and sustainable management of mescal Agaves in central Mexico: Agave potatorum in the Tehuacán-Cuicatlán Valley. *Economic Botany 69*, 26-41. https://doi.org/10.1007/s12231-014-9295-2.

Ueno, K., Sonoda, T., Yoshida, M., Shiomi, N. and Onodera, S. (2018). Purification, characterization, and functional analysis of a novel 6G&1-FEH mainly hydrolyzing neokestose from asparagus. *Journal of Experimental Botany 69*, 4295-4308. https://doi.org/10.1093/jxb/ery234.

Valdez-Cruz, N. A., Reynoso-Cereceda, G. I., Pérez-Rodriguez, S., Restrepo-Pineda, S., González-Santana, J., Olvera, A., Zavala, G., Alagón, A. and Trujillo-Roldán, M. A. (2017). Production of a recombinant phospholipase A2 in *Escherichia coli* using resonant acoustic mixing that improves oxygen transfer in shake flasks. *Microbial Cell Factories 16*, 129. https://doi.org/10.1186/s12934-017-0746-1.

Walsh, G. (2018). Biopharmaceutical benchmarks 2018. *Nature Biotechnology 36*, 1136-1145. https://doi.org/10.1038/nbt.4305.

Williams, D. P., Regier, D., Akiyoshi, D., Genbauffe, F. and Murphy, J. R. (1988). Design, synthesis and expression of a human interleukin-2 gene incorporating the codon usage bias found in highly expressed *Escherichia coli* genes. *Nucleic Acids Research 16*, 10453-10467. https://doi.org/10.1093/nar/16.22.10453.

Wu, Y., Jia, X., Huang, D., Zheng, J., Hu, Z. and Xu, C. (2019). Production, structural characterization, and antiproliferative activity of exopolysaccharide produced by Scleroderma areolatum Ehrenb with different carbon source. *Brazilian Journal of Microbiology 50*, 625-632. https://doi.org/10.1007/s42770-019-00071-9.

Zeng, H. and Yang, A. (2019). Quantification of proteomic and metabolic burdens predicts growth retardation and overflow metabolism in recombinant *Escherichia coli*. *Biotechnology and Bioengineering 116*, 1484-1495. https://doi.org/10.1002/bit.26943