Production and Viscosity of Xanthan Gum are increased by LED irradiation of *X. campestris* cultivated in medium containing produced water of the oil Industry.

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Abstract
Oil recovery is a challenge and microbial enhanced oil recovery is an option. We theorized that the use of produced water (PW) with photo-stimulation could influence both production and viscosity of Xanthan gum. This study aimed at the evaluation of the effect of photo-stimulation by λ630 ± 1 ηm LED light on the biosynthesis of Xanthan gum produced by Xanthomonas campestris IBSBF 2103 strain reusing PW of the oil industry. We assessed the effect of photo-stimulation by LED light (λ630 nm) on the biosynthesis of Xanthan gum produced by *X. campestris* in medium containing produced water. Different energy densities applied during the microbial growth phase were tested. The highest production was achieved when using 12 J/cm^2 LED light ($p<0.01$). Three protocols were assessed: Non-irradiated (Control), Irradiation with LED light during the growth phase (LED_{growth}) and Irradiation with LED light during both growth and production phases ($LED_{growth+production}$). Both the amount and viscosity of the xanthan gum was significantly higher ($p<0.01$) in the group $LED_{growth+production}$. The study showed that LED irradiation (λ630 ± 1 ηm) during both the growth and production phases of the biopolymer increased both the production and viscosity of Xanthan gum.

Keywords: Biopolymer; MEOR; Photo-stimulation; Thermoplastic property.

1. Introduction

The wide application of microbial biopolymers worldwide led to the development of techniques to optimize their production through selection of new strains, together with optimizing conditions of cell growth, production, recovery, and purification [1-5].

Xanthan gum (XG) is an exopolysaccharide (EPS) of bacterial origin used in the petrol industry due to its thermoplastic properties [6]. XG is a long polysaccharide in which the main chain consists of D-glucose molecules and a trisaccharide side chain containing two D-mannose molecules (acetylated and/or pyruvated) interspersed with one D-glucuronic acid molecule [7]. Its physicochemical characteristics may be altered when using different bacteria and/or production conditions [8-11].

Photo-stimulation is a useful technique in biotechnology as it may enhance biological processes and so assist effective production of biomass and bioenergy in an ecologically friendly and sustainable manner. Short-term irradiation of microbial cultures with laser/LED light can improve the adaptability of different strains and increase the production of bio compounds [12,13].

Produced water (PW) is one of the large-volume residual effluents generated by the oil industry. Sustainable management of it is needed so that no environmental impacts occur [14]. The global market for the treatment of PW has been estimated to be $6.2 billion in 2020 and $9.79 billion in 2024 [15]. Strict regulations regarding the disposal of this by-product have led to the development of biodegradation systems for the treatment of PW [16,17]. Our group has recently shown that the type of water used in the bacteria's culture medium affects both composition and amount of XG. The extent of XG and pyruvate production

is affected differentially in *X. campestris* when grown in distilled water and irradiated with LED versus dialyzed produced water plus LED irradiation [18]. Enhancement of XG production was observed when using distilled water in the culture medium. In contrast, both pyruvate acetyl mannose content increased in the dialyzed-produced water culture [19].

We theorized that the use of produced water (PW) in combination with photo-stimulation could influence both production and viscosity of Xanthan gum. This study aimed to evaluate the effect of photo-stimulation by λ630 ± 1 ηm LED light on the biosynthesis of Xanthan gum produced by *Xanthomonas campestris* IBSBF 2103 strain reusing PW of the oil industry.

2. Material and Methods

2.1. Microorganism and its conservation

The *Xanthomonas campestris* IBSBF 2103 strain (Culture Collection of the Biological Institute - Campinas, SP, Brazil) was used. To ensure genomic integrity and reduce the risk of fluctuations due to genetic alterations of the strain [20], a stock conservation was made in cryogenic tubes of 2 mL as previously described by our team [19].

2.2. Conditions of Growth and Production

Two hundred and twenty-five microliters of homogenized *X. campestris* culture were inoculated in 22.5 mL of YM medium and incubated in a stirring table at 28°C at 150 rpm [18,19]. After 24-h of growth, 10 mL of the culture were inoculated in 90 mL of modified MSM medium as previously described [18, 21].

157 *2.3. Dialysis of the Produced Water*

158

159 The PW employed was collected from a Brazilian carbonate oil field during
160 primary recovery activities. Its physicochemical characteristics, metal content and
161 dialysis were carried out as previously reported by our team [18].

162

163 *2.4. Parameters and Protocols of Irradiation*

164

165 *2.4.1. Determination of the light source and energy density*

166 In this study either a laser (Twin flex, MM Optics São Carlos, SP, Brazil)
167 or a LED prototype (St. Andrews University[1]) were used for irradiation that was
168 carried out in 24-wells plates in which black ink was used in the intermediate wells
169 to avoid light transmission to adjacent wells. The irradiation parameters are
170 described in *Table 1*.

171 **Table 1.** *Laser and LED emission parameters used.*

Parameter	*Laser*	*LED*
Wavelength (nm)	660	630 ± 1
Emission	CW	CW
Spot size (cm^2)	0.04	2.2
Power Density (mW)	40	41.2

172

173 The Laser and LED devices were calibrated and the light absorption by
174 the YM and MSM culture media (in dialyzed PW) were evaluated (Thorlabs Power
175 Meter Sensor PM 30, Newton, New Jersey, United States) to verify the effective
176 energy density delivered in each study group. The focal length used was 1 mm
177 with a negligible divergence angle.

178 Initially, energy densities of 4, 8, 12, 16, 20 J/cm^2 were evaluated for both
179 devices to obtain most efficient energy density as well as the light source.

[1] Organic Semiconductors Centre, Department of Physics and Astronomy, St. Andrews University High Power LED devices Cree XP emission wavelength λ630 ± 1 nm.

180 Irradiation was applied at 6 and 24-h in the growth phase of *X. campestris* in the
181 YM medium, during incubation on a stirring table at 28°C at 150 rpm (New
182 Brunswick Scientific Co I26 Incubator Shaker Series, São Diego-CA, USA) and
183 the productions in MSM medium with DPW.

184

185 *2.5. Evaluation of Photo-stimulation in Xanthan Production with DPW*

186

187 Three study groups were evaluated in triplicate: control group, irradiated
188 group in the growth of *Xanthomonas* (LED_{growth}) and the group irradiated during
189 both growth of *Xanthomonas* and production of the biopolymer ($LED_{growth+production}$)
190 (*Tab. 2*).

191

192 **Table 2.** *Irradiation Protocol of the study groups.*

Groups	*Bacterial Growth (YM) 6 and 24-h*	*Polymer Production (MSM) 6 and 24-h*
Control	_	_
LED_{Cresc}	12 J/cm^2	_
$LED_{Cresc + Prod}$	12 J/cm^2	12 J/cm^2

193

194 *2.6. Characterization of the Biopolymer by FTIR*

195

196 The Fourier model IR Prestige-21 transform infrared spectrometer
197 (Shimadzu, Kyoto, Japan) was used in the 400-4000 cm^{-1} spectral region with
198 resolution of 4 cm^{-1}. Thirty-two scans of each sample were carried out [22]. The
199 analyses were performed on 1 mg tablets of each macerated sample with 250
200 mg of KBr (spectroscopic grade) submitted to high pressure. Three spectra were
201 acquired sequentially for each sample, which were processed and analyzed with
202 *OriginPro*® 7.5 software.

203
204

205 *2.7. Quantification of Xanthomonas campestris during production by*
206 fluorochromatic staining
207
208 After 24-h of production, triplicate samples were from all study groups were
209 used for bacterial quantification by direct counting using a fluorescence
210 microscope [23].
211
212 *2.8. Quantification of XG Production*
213
214 As previously described by our team [18], after 120-h of production, the
215 contents of each Erlenmeyer were centrifuged and insolubilized. The
216 insolubilized material was then recuperated by centrifugation [18]. The gum was
217 then arranged for drying in an oven at 30°C until constant weight was achieved.
218
219 *2.9. Determination of the Viscosity of the Produced Xanthan Gum*
220
221 One percent solutions of the biopolymer produced were prepared and
222 kept at rest at 4°C for 24-h. Viscosity was analyzed (Brookfield MVD-8 digital
223 rotational viscometer, Marte científica, Santa Rita do Sapucaí, MG, Brazil), using
224 shear sensors (spindles) at constant speed and temperature of 25°C [24].
225
226 *2.10. Statistical analysis*
227
228 The analysis of the results was performed using the ANOVA statistical test with
229 Turkey's multiple comparison post-test using the GraphPad Prism® 6.0 software.
230
231 **3. Results**
232
233 *3.1. Characterization of the Produced Water*
234
235 Physicochemical analysis of the collected PW showed high salinity, and
236 the presence of metals, essentially iron, total phosphorus, and manganese
237 (*Tab.3*).

Table 3. *(**A**) Analytical characteristics of the PW; (**B**) Metals determined by Inductively Coupled Plasma Optical Emission Spectrometry -ICP-OES.*

A

Parameter	Result
Chloride (Cl^-)	77889 $mg.L^{-1}$
pH (25°C)	6,31
Salinity	41,20 $g.L^{-1}$
Total Oils and Greases	< 5$mg.L^{-1}$
Potential Oxidation Reduction	60,30 mV
Total Petroleum Hydrocarbon (TPH)	807 $\mu g.L^{-1}$
Unresolved Complex Mixture (MCNR)	595 $\mu g.L^{-1}$
Pristano	< 20$\mu g.L^{-1}$
Phytane	< 20$\mu g.L^{-1}$
Total dissolved solids	41400 $mg.L^{-1}$
Total Alkalinity	115 $mgCaCO_3L^{-1}$
Sulfate	230,05 $mg.L^{-1}$

B

Metal	Result ($mg.L^{-1}$)
Manganese	2,1
Iron	8,1
Total phosphorus	109

3.2. Determination of light emitter and energy densities

Figure 1 shows the production of XG irradiated in the growth phase by either laser or LED light, using different energy densities. It was found that the use of 12 J/cm^2 using LED light showed significant ($p<0.01$) higher production in comparison with all other groups.

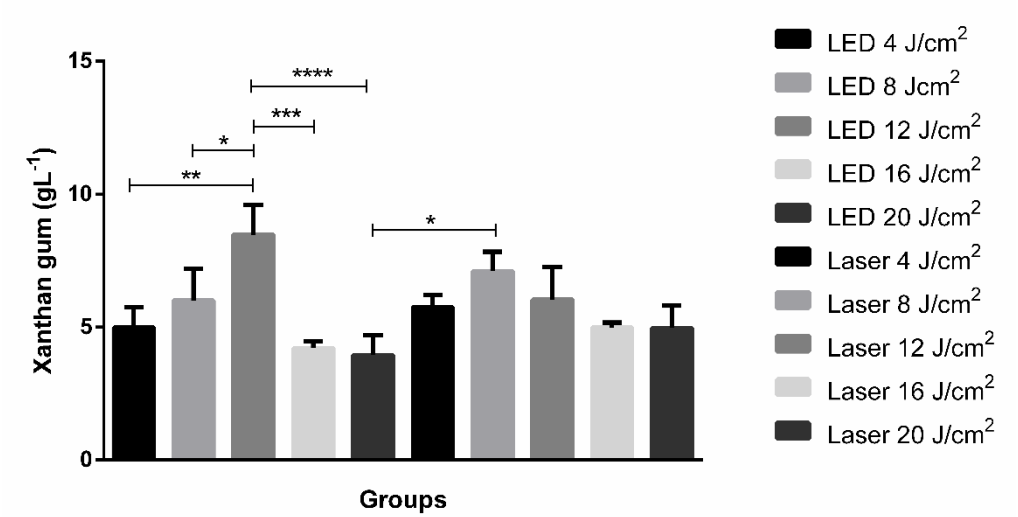
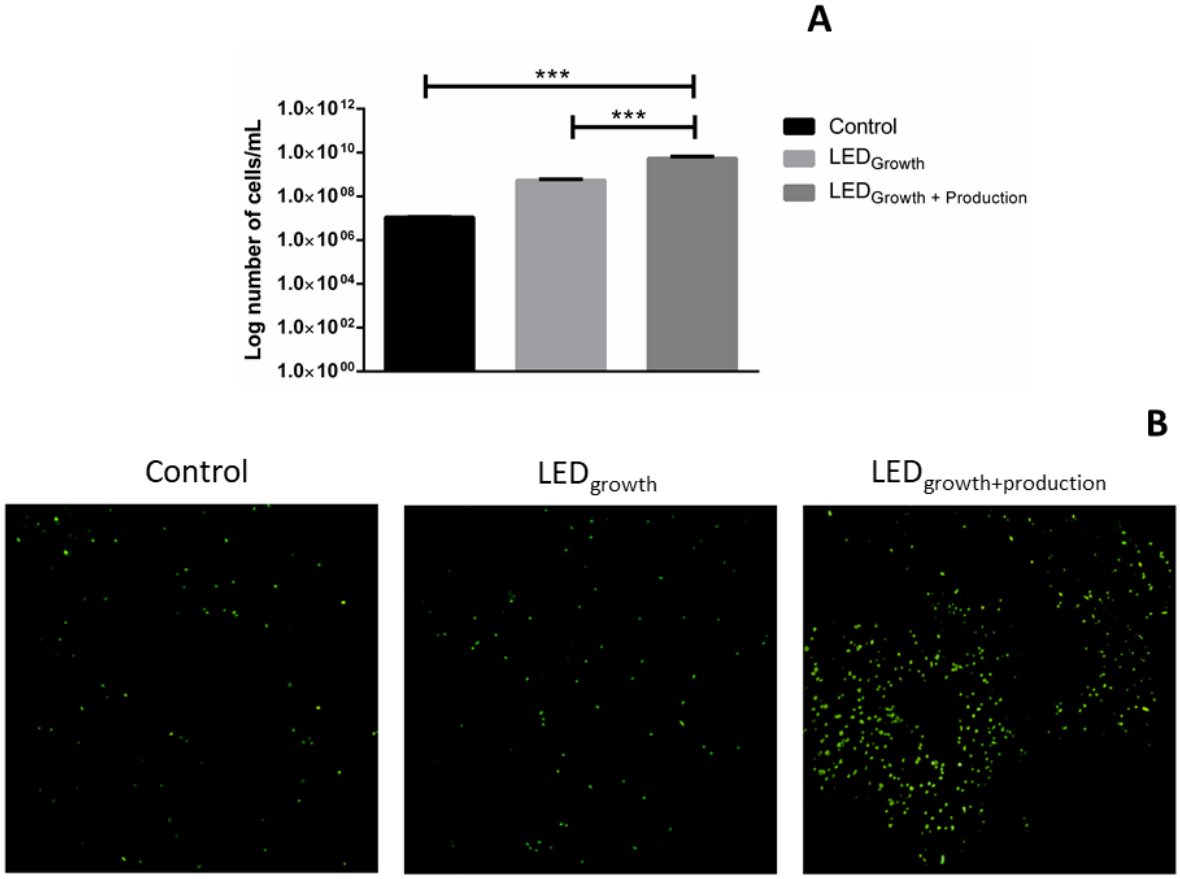


Fig. 1. *XG production of irradiated and non-irradiated X. campestris using Laser or LED light using different energy densities at 6 and 24-h of the growth phase. ****p<0.0001; ***p< 0.001; **p< 0.01; *p< 0.05.*

3.3. Evaluation of LED photo-stimulation (λ630 ± 1 ηm) in XG production

3.3.1. Quantification of Xanthomonas during Production

Cellular quantification during production indicated more X. *campestris* in the group $LED_{growth+production}$ ($p<0.001$) compared to the two other study groups. In group LED_{growth} bacterial quantification was higher than that of the control group but without statistical significance (*Fig. 2A*). The irradiation carried out during the growth and production also resulted in a positive biological response in cell proliferation (Group $LED_{growth+production}$) (*Fig. 2B*). Analysis of nucleic acids by epifluorescence there was no change in the green coloring pattern (filament) for orange (monofilament), indicating the presence of filament DNA in the study groups and absence of the process of cell division in the 24-h of production (*Fig. 2B*).



276 ***Fig. 2.*** *Fluorescence microscopy at the 24-h of production. (**A**) Statistical*
277 *analysis of bacterial quantification; (**B**) Epifluorescence of study groups*
278

279 *3.3.2. Characterization of the Biopolymer by FTIR.*

280

281 The spectra obtained by FTIR from biopolymers produced in the study
282 groups: Control, LED_{growth} e $LED_{growth+production}$, showed a broad absorption peak
283 at ~3430 cm^{-1} indicative of the elongation of the vibration of -OH group [25] and
284 typical peaks at ~1740 and ~1620 cm^{-1} representative of the stretch of carbonyl
285 (C=O) acetyl as well as the asymmetric group stretch C=O of the pyruvate
286 groups, respectively [26, 27]. Spectra also revealed absorption peaks at ~1411
287 and ~1043 cm^{-1} attributed to symmetrical stretching of the group $-COO^-$
288 glucuronic acid stretching C-O-C of the ether group, respectively [28]. Broadband
289 between ~2800 and ~2980 cm^{-1} was attributed to antisymmetric regions and
290 stretch C-H symmetrical Methylene [29], this band presents a small displacement

291 in the group $LED_{growth+production}$. On the spectrum of the $LED_{growth+production}$ group
292 there was a displacement of the absorption peak from around ~1740 to ~1725
293 cm^{-1}, while the absorption peak near ~1650 cm^{-1} shifted to ~1620 cm^{-1}. In any
294 case, the results described characterize the biopolymer as XG (*Fig. 3*).

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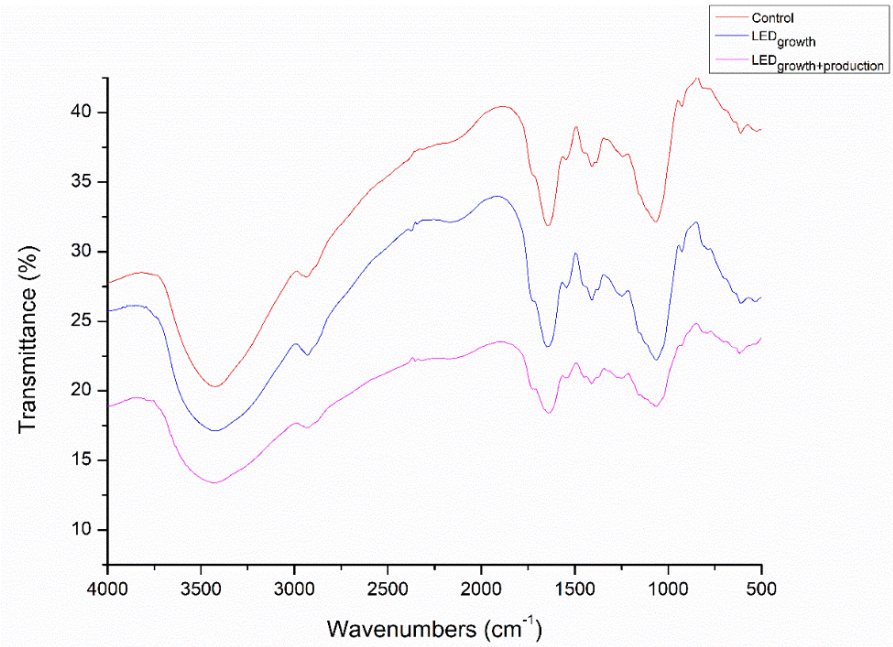

313 ***Fig. 3.*** *Infrared spectra of gums in the three study groups obtained with PW.*
314

315 *3.3.3. Quantification of Xanthan Gum Production*

316
317 The production of XG was higher in the $LED_{growth+production}$ (11.0 gL^{-1}) group
318 being 27.18 % higher than the control ($p<0.01$). In the group LED_{growth} production
319 of 10.38 gL^{-1} was obtained being this 21.45 % higher than the control (8.55 gL^{-1},
320 $p<0.01$) (*Fig. 4*).

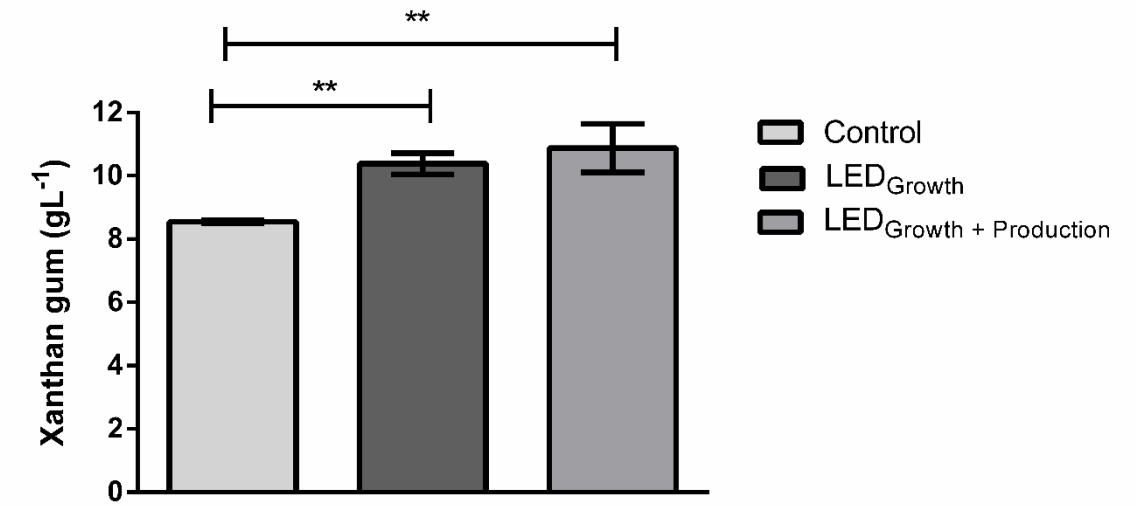

Fig. 4. *Xanthan gum production in the study groups. **p< 0.01.*

3.3.4. Viscosity Analysis of the XG

The viscosity presented in the $LED_{growth+production}$ was 599.6 mPa being significantly higher (60.2 %, p<0.01) compared to the control Group (373.2 mPa). Despite a 497.7 mPa viscosity being determined in the group LED_{growth} (33 % higher than the one of the group control) there was no statistical difference between them (*Fig. 5*).

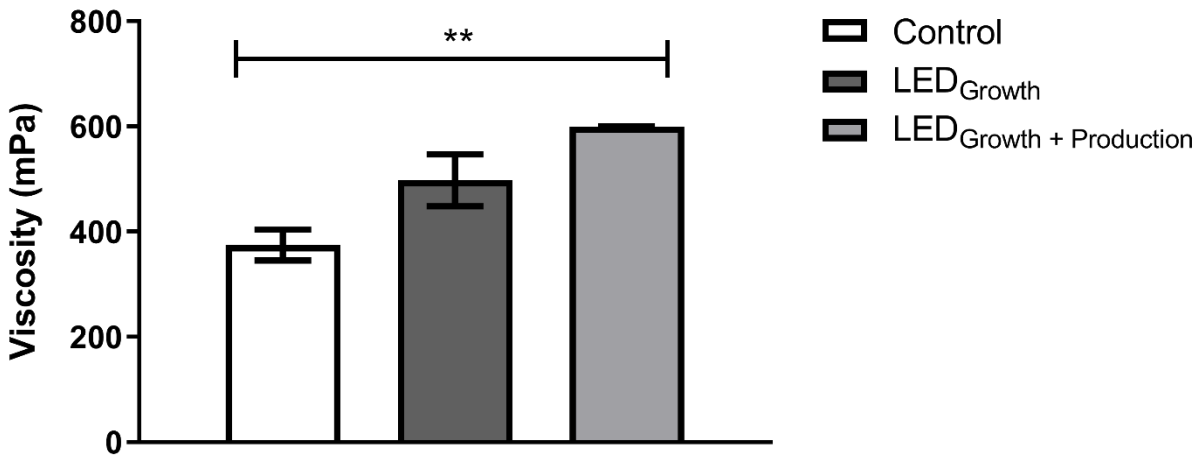

Fig. 5. *Comparison of viscosity of the XG produced in the study groups. **p<0.01*

338 **4.Discussion**

339

340 Studies have shown that salinity affects the growth of *Xanthomonas* spp.
341 and other bacteria. *Xanthomonas* spp. grows only in media with up to 3 % salinity
342 [30]. PW dialysis was performed to lower the salinity of the medium as *X.*
343 *campestris* does not grow in halophilic environments [18].

344 The energy density of light is a parameter of relevance to achieve the
345 desired photochemical effect and may be the borderline amongst the stimulatory
346 or inhibitory response [33]. Studies carried out by our team found a more
347 effective stimulatory action of the LED light (λ630 ± 1 ηm) on bacterial proliferation
348 [34, 35].

349 One of the targets of irradiation in the red spectrum is the cytochrome C
350 protein complex present in bacteria which when photo-stimulated increases its
351 proton pumping capacity, and thus the amount of cellular ATP available [36]. One
352 feature that may explain a greater efficacy of LED irradiation was demonstrated
353 previously by Karu [33] who verified that the microbial cytochrome presents its
354 maximum absorption peak at λ633 ηm, like the wavelength of the LED used on
355 this study.

356 Irradiation can also act on Zn and/or Cu atoms, found in metallic protein
357 centers or even acting as enzymatic cofactors. These metals, especially Zn, are
358 abundant in microorganisms and the metalloproteins that contain them are
359 commonly associated with transcriptional events. Thus, increased production of
360 biopolymer using adequate irradiation energy densities in relation to the control
361 group may be associated with the activation of metalloproteins [37-39]. FTIR
362 analysis showed characteristic bands of XG [22, 24]. The absorption band
363 identified at ~3430 cm^{-1} was wider in the irradiated groups, corresponding to the

364 axial deformation of hydroxyl groups with intramolecular and intermolecular
365 hydrogen limits. The displacement of the bands from ~1740 to ~1725 cm^{-1} and
366 from ~1650 to ~1620 cm^{-1}, may indicate the possibility of a coordination of the -
367 COO group with the metals from the PW [40]. Kang *et al*. [22] performed the
368 coordination of trivalent iron ions with Xanthan gum and found, by FTIR analysis,
369 that the hydrogels formed presented the same displacement observed in the
370 present study.

371 Metallic elements identified in the PW may act positively on the growth and
372 production of Xanthan gum (enzymatic cofactors). Li *et al* [31], suggested that Mg
373 ions at concentrations of up to 55 mgL^{-1}, play a role on growth, virulence
374 expression, homeostasis, and oxidative stress protection. The concentration of
375 Mg in the PW used was 2.1 mgL^{-1} and may have benefited the production of XG.

376 Study by Ciesielski and Tomasik [32] showed that the carboxylic groups of
377 XG are preferably involved in the binding of central atoms and that Cu (II), Co (II)
378 and Fe (III) ions are easily synchronized by the XG to form Wener-type
379 complexes. In the present study the PW used contained several metals in its
380 composition and these metals could have formed Wener-type complexes with
381 polysaccharide. The metals present in the PW may have been activated by LED
382 irradiation during the production phase and formed Werner-type metal
383 complexes, central atoms with the binding polysaccharide. The carboxylic groups
384 of the XG will rather be involved in the binding to the central atoms (*Fig. 6A*). Most
385 of the complexes formed between the XG and metals are mainly octahedral, but
386 also form square plane complexes [32].

387

388 The irradiations applied at the beginning and end of the exponential growth
389 phase of *Xanthomonas* culture (before the productive phase of gum) aimed at an
390 increase in the number of bacteria available for the gum production phase. There
391 is a widespread photobiological mechanism of light action in the respiratory chain
392 of eukaryotic and prokaryotic cells with terminal enzymes of the respiratory chain,
393 acting as photoreceptors [41] (*Fig. 6B*). The metabolic routes suggested by
394 Crugeira and collaborators [35] underlie the results obtained in this phase of the
395 study.

396 Light basically functions as a "trigger", that turns on primary cellular
397 responses in the respiratory chain or cell membrane, which spread through
398 successive secondary reactions in the cytoplasm and nucleus of cells, triggering
399 a cascading process [33]. Photo-stimulation causes positive responses at the
400 cellular level, such as increased proliferation rate and regulation of the expression
401 of metabolic and genetic pathways [35, 42], increased rate of RNA and DNA
402 synthesis [41] as well as ATP synthesis [43].

403 The irradiations carried out during the Xanthan gum production in the
404 $LED_{growth+production}$ group may have helped the excitation of prosthetic groups,
405 oxidoreduction mediators and activate metalloproteins that participate in the
406 catalytic action of pentasaccharide polymerization, excretion, and polysaccharide
407 formation (*Fig. 6C*) [44]. Photon absorption by prosthetic groups and/or aromatic
408 peptide residues may increase anabolic capacity or help maintain the three-
409 dimensional conformation of enzymes making them more effective [45]. For
410 polysaccharide polymerization, polysaccharide ATP is required, which may result
411 from photoactivation of cytochrome C oxidase or cytochrome bd of the respiratory

chain [33]. Effects produced by light irradiation in the activation of transcriptional and translational processes were also previously described [46,47].

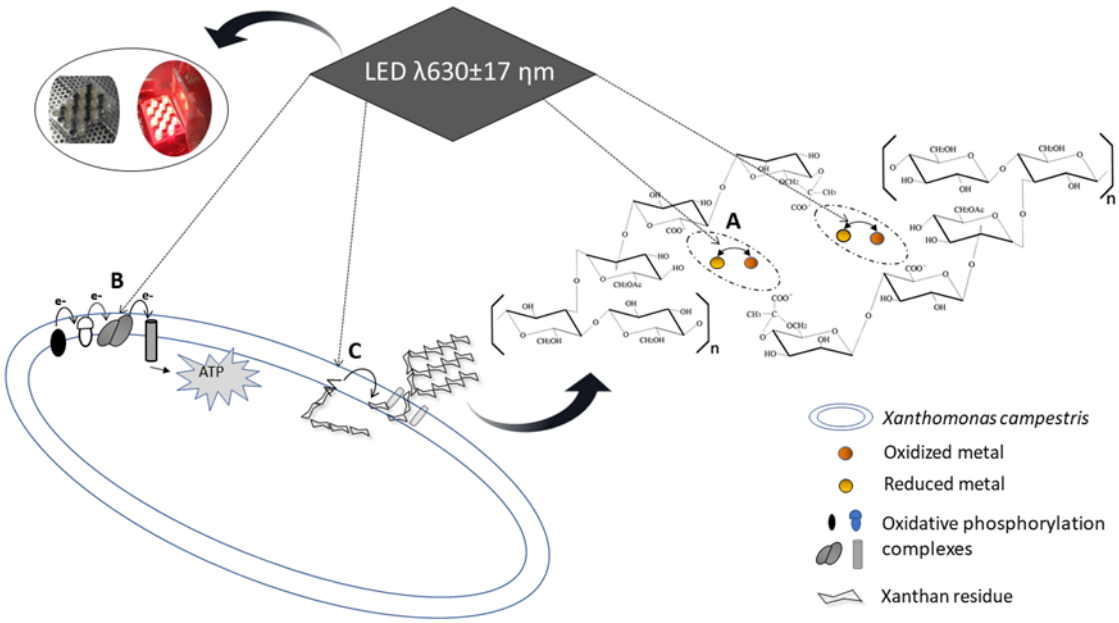

Fig.6. A representative scheme of metabolic pathways that may have been activated by LED light (λ630 ± 1 ηm) in Xanthan growth and production. (**A**) photoexcitation of metal groups during the formation of Xanthan gum, forming Wener complexes. (**B**) photoactivation of respiratory chain cytochrome oxidase. (**C**) photoexcitation of prosthetic groups that participate in the catalytic action of the polymerization of pentasaccharides, excretion and formation of the polysaccharide.

Correlating the viscosities obtained, we can conclude that the biopolymer produced in the $LED_{growth+production}$ group presented a higher quality [48,49]. Diaz *et al.* [50] associated the XG´s quality index obtained by different strains of *X. campestris* pv *pruni* with the apparent viscosity presented by the polymer [49]. The viscosity of a polysaccharide is associated to the integrity of its molecule, which, in turn, depends on its primary and secondary structure, which is directly associated with the operational conditions of the production process [51].

The content of pyruvate and acetate groups in polymers affects the intermolecular interactions of gum and between Xanthan and other polymers influencing the viscosity of the final product [9, 52]. As the production conditions

434 were maintained for the different study groups (agitation, aeration, pH,
435 temperature, and microbial strain used) it can be inferred that irradiation
436 promoted the intermolecular association, through the negative charges of the
437 Xanthan molecule with metal ions (especially trivalent) from PW, thus increasing
438 the viscosity of the obtained product [22,53].

439 A previous report using Raman spectroscopy from our group has shown
440 positive photo-induced effects on *X. campestris* [19]. The study of Xanthan gum
441 productions either using distilled water or DPW and irradiated or not by either
442 Laser (λ = 660nm) or LED (λ = 630nm ± 2nm) were indicative of increased
443 amounts of pyruvyl-mannoses in Xanthan gum produced with DPW [18,19]. It
444 was also found that photo-stimulation caused an additional increase in the
445 pyruvylation of mannose residues, thus, the increase in viscosity obtained in this
446 study in the groups irradiated by LED may be a result of such modifications (*Fig.*
447 *5*).

448 Xanthan gum solutions are characterized by non-Newtonian and highly
449 pseudoplastic, i.e., viscosity decreases with increasing fluid deformation rate
450 [54,55]. Its branched structure and high molecular weight give XG a high
451 viscosity, even at low concentrations, making it an extremely important property
452 for industry.

453 The results of the present study open up a wide field of opportunities for
454 the use of photo-biomodulation in microbial cultures enriched with PW to enhance
455 bacterial production of Xanthan gum that can be of great benefit in the use of
456 sustainable practices for oil extraction or for the industrial sector in general.

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5.Conclusion

The study showed that LED irradiation (λ630 ± 1 ηm) during both the growth and production phases of the biopolymer increased both the production and viscosity of Xanthan gum.

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