Environmental Engineering and Management Journal *June 2018, Vol.17, No. 6, 1329-1335*
http://www.eemj.icpm.tuiasi.ro/; http://www.eemj.eu

"Gheorghe Asachi" Technical University of Iasi, Romania

OPTIMIZATION OF WASTEWATER BASED MEDIA FOR BIOPOLYMERS PRODUCTION BY *Rhizobium leguminosarum*

Mohamed Sellami[1], Fakher Frikha[1], Achraf Aribi[1], Imen Taktak[1], Nabil Miled[1], Faouzi Ben Rebah[2,3]*

[1]Laboratoire de Biochimie et de Génie Enzymatique des Lipases, ENIS, Université de Sfax, Sfax- Tunisia
[2]Higher Institute of Biotechnology of Sfax, Université de Sfax, Sfax--Tunisia
[3]Department of Chemistry, College of Science, King Khalid University, Abha, Saudi Arabia

Abstract

The present work aimed to optimize a new economic medium for biopolymers production by *Rhizobium leguminosarum*. Biopolymers were poly-β-hydroxybutyrate and exopolysaccharide (EPS and PHB). They were extensively used in various industrial sectors. Statistical experimental designs and Response Surface Methodology were employed to optimize the medium components. A central composite design was applied to increase the production yield and predict the optimal values of the selected factors. An optimal medium, for PHB and EPS production of about 75.12 ± 5.87 mg/L and 9.2 ± 0.66 g/L, respectively, was found to be composed of 10 g/L of sucrose and 3 g/L of yeast extract added to the wastewater based medium. These two components were added to the wastewater to accurate the nutrient composition of the medium growth. This work shows for the first time the feasibility of using rhizobial strains growing in industrial wastewater to coproduce EPS and PHB.

Key words: agro-industrial wastewater, exopolysaccharide, poly-β-hydroxybutyrate, *Rhizobium leguminosarum*

Received: April, 2014; Revised final: July, 2014; Accepted: August, 2014; Published in final edited form: June 2018

1. Introduction

There has been considerable interest in the development and production of biodegradable polymers from renewable sources. Particular attention was devoted to microbial polymers due to their great variation that allows the continuous increase in the search for product formulation useful in numerous industrial sectors (Villano et al., 2014; Wang et al., 2012). Bacterial polymers have particularly attractive properties (non-toxicity, biocompatibility, biodegradability, selective permeability, physico-mechanical properties, etc.) and could be a solution to the environmental problems caused by the waste generated by the petroleum based polymers. Among the bacterial polymers, polyhydroxybutyrate (PHB) and exopolysaccharides (EPS) are extensively used in various industrial sectors. PHB is known to be accumulated as intracellular inclusion in some bacteria as carbon and energy reserves (Grothe et al., 1999; Liu et al., 1998; Tavernier 2008), which can be used mainly in biodegradable plastics production and pharmaceutical sector (Singh et al., 2011). However, the EPS polymers are synthesized inside the microbial cell and excreted in the medium in the form of macromolecules (Castellane et al., 2015; Kumari et al., 2009; Sutherland, 1985). Because of their different properties, EPS found applications in many industrial sectors such as medicine, agriculture and food industries (emulsifiers, stabilizers, binders, gelling agents, coagulating agents, flocculating agents, film-forming substances, lubricants, thickening agents, immunostimulating agents and antitumor agents etc.) (Donot et al., 2012).

* Author to whom all correspondence should be addressed: Email: benrebahf@yahoo.fr

Rhizobia are able to synthesize both EPS and PHB (Fouchet et al., 1995; Stam et al., 1986; Sutherland 1985; Tavernier et al., 1997). Rhizobial EPS are implicated in the *Rhizobium*-legume symbiosis and are considered as important factors for symbiotic efficacity (Breedveld and Miller, 1998; Fraysse et al., 2003). They act as messengers during host plant infection. These molecules are needed for the continuous development of the infection thread allowing the formation of biofilm on abiotic surfaces and on roots of the host plants (Gonzalez et al., 1996; Rinaudi 2009; Xie et al., 2012).

The rhizobial EPS biosynthesis represents a multi-step process controlled by a complex protein located on both inner and outer bacterial membranes. The exopolysaccharide properties (structure, composition, viscosity etc.) depend on several factors related to the growth environment (nitrogen, carbon, mineral salts, pH temperature, aeration etc.) (Duta et al., 2006; Janczarek, 2011). PHB is synthesized under specific conditions as intracellular carbon and energy storage polymer (Fouchet et al., 1995; Stam et al., 1986).

The biosynthesis of polymers by *Rhizobia* controlled by the genetic characteristics and the culture conditions (the nutrient sources, the nature and the availability of carbon and nitrogen sources, pH, temperature, oxygenation, concentrations) (Belal, 2013; Duta et al., 2006; Tavernier et al., 1997). Besides, to produce microbial polymers, a wide variety of raw material has been used as growth medium for various microbial strains.

The carbon source is an important factor that can influence the growth of microorganisms and the polymer production. Nevertheless, the price and availability of these substrates can be associated with the production cost. Different studies have been conducted in order to study the impact of the concentration and the type of carbon source on the PHB and EPS production (Ben Rebah et al., 2009; Cerning et al., 1994; Gamar et al., 1997). The recovery of waste is a beneficial solution to ensure environmental quality. Particularly, the challenge for wastewater treatment plants is to find ways to reduce the cost of the treatment process and the sludge disposal.

Because wastewater and sludge contain various nutrients (carbon, nitrogen, phosphorus, potassium and magnesium etc.) useful for microbial growth, they have been used as growth media for various rhizobial stains (Ben Rebah et al., 2001; 2002; 2009; Yan et al., 2006). However, no studies have reported the use of wastewater as a carbon source for growing rhizobial strains in order to produce simultaneously EPS and PHB. Therefore, the objective of this work was to maximize EPS and PHB production by *Rhizobium leguminosarum* cultivated in industrial wastewater. Carbon and nitrogen sources added to the wastewater were optimized using central composite design.

2. Experimental

2.1. Wastewater sampling and characterization

Food processing wastewater was collected from confectionery factory (Le Moulin-Triki, Sfax-Tunisia), which generates a substantial volume of wastewater of 30 m^3/day. The wastewater was analyzed and stored at 4°C until their use (APHA 1992). Wastewater characteristics are shown in Table 1.

Table 1. Wastewater characteristics

Parameters	***Value***
pH	4.94
SS: Suspended solids (mg/L)	230
COD: Chemical Oxygen Demand (mg/L)	2376
BOD_5: Biochemical Oxygen Demand (mg/L)	1550
TKN: Total Kjeldahl Nitrogen (mg/L)	140

2.2. Microorganism

A strain of *Rhizobium leguminosarum* (ATCC 10004) was used during the experiments. The inoculum was prepared in Yeast Mannitol Broth (YMB) as described by Ben Rebah et al. (2009)

The growth of the strain was conducted in wastewater filtrated under vacuum (to eliminate suspended solids) and supplemented with various concentrations of sucrose as carbon source and yeast extract as a nitrogen source. Samples for rhizobial growth were prepared as described by Ben Rebah et al. (2009) and inoculated with inoculum size of 4% v/v. Growth experiments were stopped after 72 hours.

2.3. Determination of EPS

The quantity of exopolysaccharide produced by the strain in the growth medium was determined as described by Damery and Alexander (1969) and Kumari et al. (2009).

2.4. Determination of PHB

A sample of culture medium was centrifuged for 10 min at 8000 rpm and washed twice with distilled water. Then, the pellet was treated as described by Anderson and Dawes (1990). The obtained cell residue was subject to extraction with chloroform and PHB was evaluated using the crotonic acid method (Law and Slepecky 1960).

2.5. Experiment design and statistical analysis

Five-level ($-\alpha$, -1, 0, +1, $+\alpha$), two-factors (sucrose and yeast extract concentrations) Central Composite Design (CCD) was applied in this study. With two factors, the total number of runs is equal to 12; 2^2 (two level factorial) + 2×2 (axial points) + 4 (center points) = 12 (Myers and Montgomery 1995).

The two-level factorial part of the design consists of all possible combinations of the +1 and –1 levels of the coded factors (4 design points: (1, 1), (1, -1), (-1, 1) and (-1, -1)). Axial points are (-α, 0), (α, 0), (0, α) and (0, -α). Center points : data from the center points provides estimates of pure error and estimates of curvature. The coded and uncoded variables and their respective level are shown in Table 2.

Design Expert 7 was used to analyze the CCD results. The effects of sucrose and yeast extract concentrations on PHB and EPS yields were evaluated. The interaction of the two variables was also calculated. ANOVA test was used to evaluate the significance of variables. A mathematical correlation associated the response (PHB or EPS) to the two variables (sucrose and yeast extract) can be presented by an equation. This mathematical model should be evaluated and validated (at 95% of confidence level).

The regression models of two responses (PHB and EPS) were established through second order polynomial equation and were presented as follows (Eq. 1):

$$Y = a_0 + a_1X_1 + a_2X_2 + a_{12}X_1X_2 + a_{11}X_1^2 + a_{22}X_2^2 \quad (1)$$

where: Y is the response (PHB and EPS production), X_1 and X_2 are the input variables (sucrose and yeast extract concentrations), a_0 is intercept term, a_1, a_2 are linear coefficient, a_{11}, a_{22} are quadratic coefficient and a_{12} is interaction coefficient.

3. Results and discussion

The wastewater used in the present study is from a confectionery (Triki-Le Moulin, Sfax). It mostly contains the nutrients (Table 1) that support the microbial growth and/or bio-product formation (PHB and EPS). In preliminary study, it was demonstrated, that the wastewater can be used as growth media for *Rhizobium leguminosarum* and during growth substantial quantities of PHB and EPS were produced by the strain. Growing in this wastewater, PHB and EPS yields did not exceed 8.55 mg/L and 4.40 g/L respectively (data not shown).

These results confirmed those reported by Ben Rebah et al., (2001; 2002; 2009) concerning the possibility of growing Rhizobia in wastewater and in sludge. Generally, the media used for biopolymer production, in laboratory studies, may vary considerably from industrial production media. In laboratory studies, pure substrates such as glucose, sucrose, and glycerol can be used to determine yields. In industrial production, the main factors that control the choice of the substrate to be used are the substrate cost, the yield and the quality of the product. In order to enhance rhizobial PHB and EPS productivities in wastewater used in this study, the effect of sucrose and yeast extract were chosen as nutrients sources. Our strategy can lower the cost of growth media for biopolymer production and reduce the industrial wastewater handling and disposal. This can provides an environmental sound way of wastewater management and use in diverse biotechnological application. A central composite design was used to optimise levels of sucrose and yeast extract, added to the wastewater, to maximize production of PHB and EPS by *Rhizobium leguminosarum*. Table 2 shows the coded and the real experimental conditions of the mixture design with the corresponding measured responses. The independent variables and runs were generated by Design-Expert Software. The quadratic model was found to be the best fitted to the responses. A second order polynomial equation was employed to fit the experimental data presented in Table 2.

Table 2. Central composite design matrix with the observed and predicted values

	Experimental Condition		***Responses***			
	Nutrient source (g/L)		***PHB (mg/L)***		***EPS (g/L)***	
Run	***Sucrose (X_1)***	***Yeast Extract (X_2)***	***Observed Value***	***Predicted value***	***Observed value***	***Predicted value***
1	2.00 (-1)	1.00 (-1)	29.09	20.94	6.8	6.98
2	10.00 (+1)	1.00 (-1)	19.43	23.82	7.7	8.38
3	2.00 (-1)	3.00 (+1)	69.75	55.14	5.3	5.67
4	10.00 (+1)	3.00 (+1)	75.12	73.06	9.2	10.12
5	0.34 (-1.41)	2.00 (0)	18.24	32.05	7.3	7.00
6	11.66 (+1.41)	2.00 (0)	48.29	46.77	9.6	11.15
7	6.00 (0)	0.59 (-1.41)	16.22	17.73	5.9	6.35
8	6.00 (0)	3.41 (+1.41)	66.05	76.56	6.4	6.66
9	6.00 (0)	2.00 (0)	68.20	65.04	7.6	7.22
10	6.00 (0)	2.00 (0)	70.58	65.04	7.2	7.22
11	6.00 (0)	2.00 (0)	62.60	65.04	6.2	7.22
12	6.00 (0)	2.00 (0)	57.47	65.04	6.4	7.22

The response data (PHB and EPS production) in Table 2 was converted into two polynomial equations with two independent variables. Consequently, the polynomial models describing the correlation between responses and variables were (Eqs. 2, 3):

$$Y_{EPS\ (in\ g/L)} = +7.73 - 0.71X_1 + 0.41X_2 + 0.19\ X_1X_2 + 0.05\ X_1^2 - 0.36\ X_2^2 \quad (2)$$

with adjusted $R^2 = 0.83$

$$Y_{PHB\ (in\ mg/L)} = -38.00 + 9.02X_1 + 51.22\ X_2 + 0.94\ X_1\ X_2 - 0.80\ X_1^2 - 9.00\ X_2^2 \quad (3)$$

with adjusted $R^2 = 0.77$

where: Y_{EPS} and Y_{PHB} are the predicted responses of EPS and PHB production, respectively; X_1 and X_2 are the concentrations of sucrose and yeast extract, respectively.

ANOVA was also performed (Table 3). The values of R^2, a measurement for fitness of the regressed Eq. (2) and Eq. (3) were 0.91 and 0.88, respectively. These resultants indicated that the experimental data were in a good agreement with predicted values. The associated *p*-value was used to estimate whether *F*-value was large enough to indicate statistical significance. A *p*-value below 0.05 indicates that the model was statistically significant. The PHB produced by Rhizobium increased with the enhancement of both the sucrose and yeast extract concentrations added to the growth medium (Fig. 1). For yeast extract concentrations superior to 2 g/L, a significant enhancement of PHB yield was observed. Generally, PHB synthesis can be selectively induced in Rhizobium strains by sources of carbon and nitrogen and the nature of nutrient sources may affect the PHB rates. For example, the culture of *R. phaseoli* 680 in the presence of sucrose and nitrate may allow PHB yield up to 65% of dry cell weight (Bonartseva et al., 1994). This illustrates that nitrate is an importance factors in the biopolymer accumulation by Rhizobium. In our study, only the total Kjeldahl nitrogen was determined for the used wastewater. Therefore, it seems interesting to determine the different nitrogen forms contained in this media such as nitrate and ammonia, allowing the investigation of the significant nitrogen sources that enhanced polymer production by the rhizobaial strain. Furthermore, the C/N in the wastewater may have an impact on PHB accumulation as reported by Ben Rebah et al., (2009) while growing *S. meliloti* in starch and slaughterhouse wastewaters.

Table 3. ANOVA and regression analysis of surface quadratic model for EPS and PHB

Source	*Sum of Squares*	*Degrees of freedom*	*Mean Square*	*F-value*	*p-value*	
PHB						
Model	5062.85	5	1012.57	8.57	0.0105	Significant*
X_1	182.40	1	182.40	1.54	0.2605	
X_2	3478.64	1	3478.64	29.43	0.0016	
$X_1\ X_2$	56.42	1	56.42	0.48	0.5155	
X_1^2	1071.07	1	1071.07	9.06	0.0237	
X_2^2	518.62	1	518.62	4.39	0.0811	
Residual	709.31	6	118.22			
Lack of Fit	605.72	3	201.91	5.85	0.0905	not significant
Pure Error	103.59	3	34.53			
Cor Total	5772.15	11				
R-Squared = 0.88						
EPS						
Model	16.19	5	3.24	11.59	0.0049	Significant*
X_1	8.11	1	8.11	29.00	0.0017	
X_2	0.06	1	0.06	0.22	0.6530	
$X_1\ X_2$	2.25	1	2.25	8.05	0.0297	
X_1^2	3.97	1	3.97	14.20	0.0093	
X_2^2	0.84	1	0.84	3.01	0.1335	
Residual	1.68	6	0.28			
Lack of Fit	0.37	3	0.12	0.28	0.8382	not significant
Pure Error	1.31	3	0.44			
Cor Total	17.87	11				
R-Squared = 0.91						

*Statistically significant at 95% of confidence level

Fig. 2 showed that an increase of sucrose concentration from 2 to 10 g/L and using a yeast extract concentration more than 2 g/L improved the EPS production. Generally, carbon is the most important component of the media used for the production of EPS because it directly affects the production yields, compositions, structures and properties of bacterial exopolysaccharide (Fialho et al., 1999). However, a limit nitrogen concentration allows an excess of EPS production for some Gram-bacterial strains such as *Xanthomonas*, *Pseudomonas* and *Rhizobium* (Sutherland, 1990). As reported for PHB production, the C/N ratio is very important and an abundant secretion of EPS is usually most noticeable when bacteria are supplied with abundant carbon source and minimal nitrogen (Pollock, 2002).

The addition of yeast extract in the growth medium could have a beneficial effect on rhizobial growth and on polymer production, because it supplies the growth medium with various compounds and might be used as a carbon and nitrogen source by rhizobia (Meade et al., 1985). Yeast extract contains, amino acids, inorganic nitrogen and growth factors (iron, calcium, magnesium, strontium, sodium, potassium, barium, manganese, copper, lead, aluminium and vanadium) at a concentration that is enough for nutritional requirement of rhizobial growth (Burton, 1979). A better rhizobial growth is usually obtained when low molecular weight amino acids are added (Burton, 1979). In many research activities vitamins and amino acids were used as nitrogen source or as stimulator for improving gellan gum production (Ashtaputre and Shah, 1995; Giavasis et al., 2000; Nampoothiri et al., 2003). However, the use of high concentration of yeast extract cannot be suitable for large industrial production due to the economic consideration. Moreover, the use of yeast extract concentration higher than 0.35% is not beneficial for rhizobial growth because it produces distorted cell and decreases the viability for some strains (Skinner et al., 1977). The optimal conditions for EPS or PHB coproduction were predicted using the optimization function of the Design Expert Software. To improve the economic competitiveness of the wastewater used as growth medium and maximizing EPS and PHB coproduction, sucrose and yeast extract were added. These goals were satisfied by adding nutrient source levels given in Table 4. These solutions provide EPS and PHB yields of 9.12 g/L and 77.71 mg/L respectively. Experiments were conducted under optimal conditions in order to assess the validity of regression models (Table 4).

The result demonstrated that the experiment data were in good agreement with the predicted values confirming the validity and the adequacy of the predicted models. The highest EPS (9.2 ± 0.66 g/L) and PHB production (75.12 ± 5.87 mg/L) were obtained with the same growth media (10 g/L of sucrose and 3 g/L of yeast extract). For an industrial point of view and to minimize the cost efficiency of the growth medium for PHB production, a high yield of 66.77± 5.87 mg/L could be obtained when using 7.51 g/L of sucrose and 2.95 g/L of yeast extract.

4. Conclusions

Utilization of industrial wastewater provides alternative substrates for biopolymer production and may help solving pollution problems, which otherwise might be caused by their disposal and treatment.

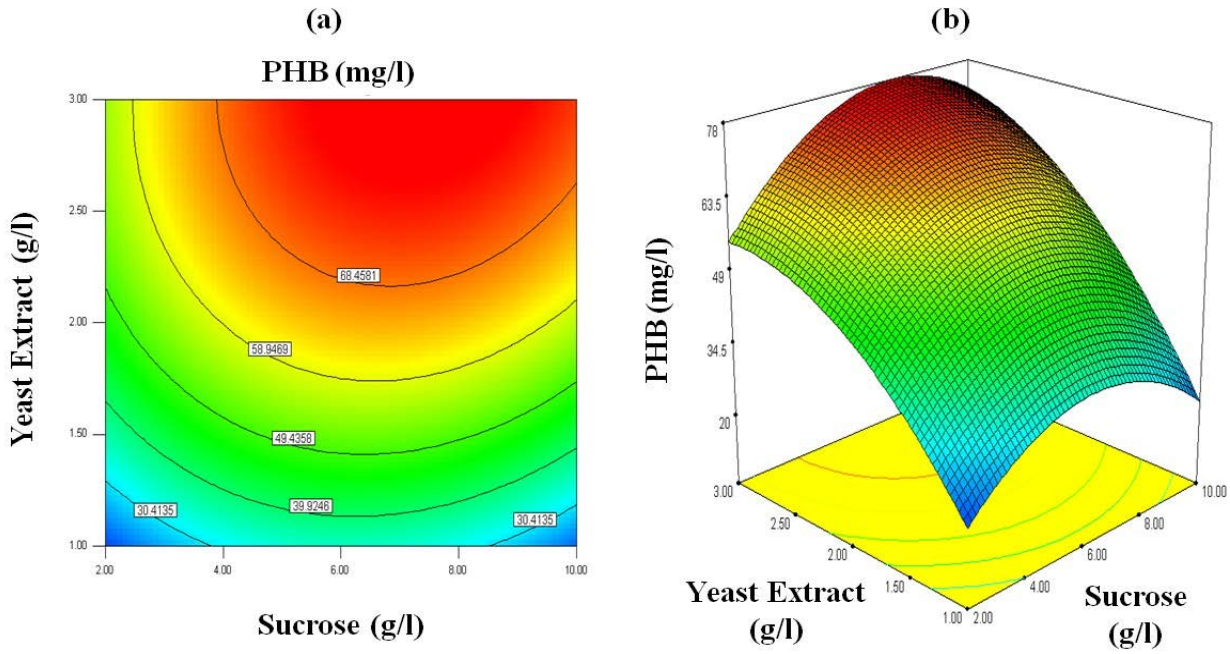

Fig. 1. Contour diagrams (**a**) and response surface (**b**) for the PHB produced by *Rhizobium* as a function of the concentrations of sucrose and yeast extract added to the wastewater

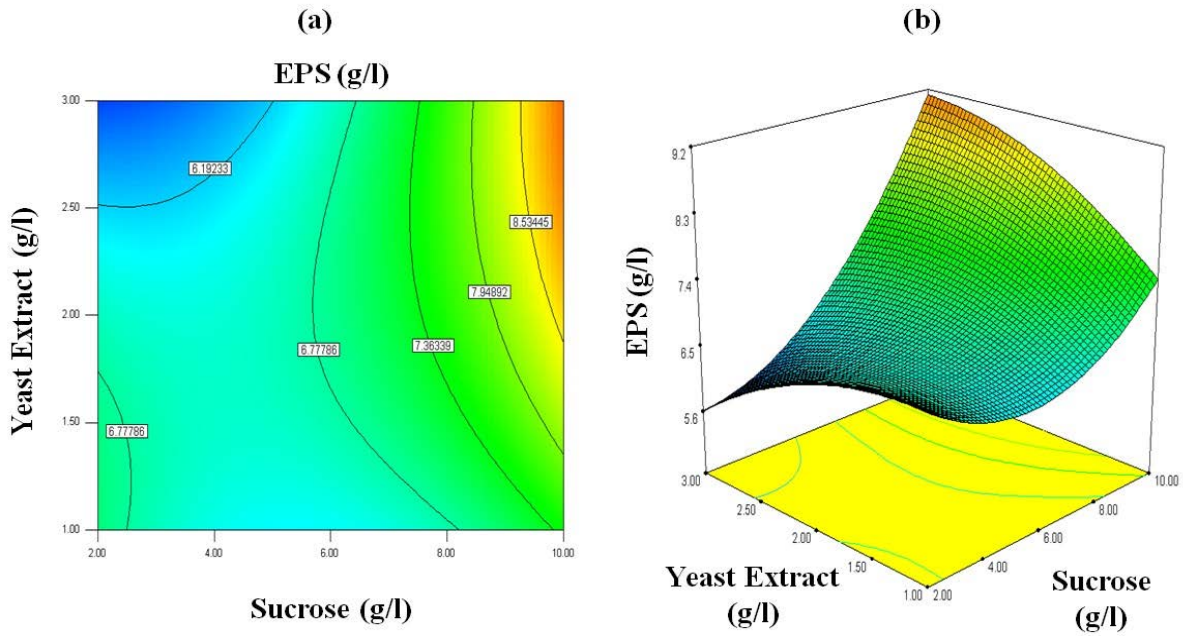

Fig. 2. Contour diagrams (**a**) and response surface (**b**) for the EPS produced by Rhizobium as a function of the concentrations of sucrose and yeast extract added to the wastewater

Table 4. Solutions for optimal conditions as generated by the Design Expert Software

Experimental Condition		***Response***			
Nutrient source (g/L)		***PHB (mg/L)***		***EPS (g/L)***	
Sucrose (X_1)	*Yeast Extract (X_2)*	*Observed value*	*Predicted value*	*Observed value*	*Predicted value*
10*	3*	75.12 ± 5.87	72.16±10.87	9.2 ± 0.66	9.12 ± 0.53
7.51	2.95	66.77± 5.87	77.71 ±10.87	-	-

**Concentrations maximizing PHB and EPS productions at the same time*

Wastewater was the most important factor affecting both EPS and PHB production. In this work, it was demonstrated that central composite design was a reliable method for determining the nutrient concentrations supplemented to the growth medium in order to maximize EPS and PHB yields.

However, the predicted optimum conditions should be verified in large scale. Also, other factors could be optimized using the CCD (such as salts, trace element, pH, aeration, agitation etc). The screening of other waste as nutrients sources (fish processing waste as nitrogen source, etc.) should be also examined.

Acknowledgements

This work received financial support from "Ministère de l'enseignement supérieur et de la recherche scientifique, Tunisie" granted to the Laboratoire de Biochimie et de Génie Enzymatique des Lipases.

References

Anderson A.J., Dawes E.A., (1990), Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates, *Journal of Microbiology*, **4**, 450-472.

APHA, (1992), Standard Methods for the Examination of Water and Wastewater. 18th edition, American Public Health Association (APHA), American Water Works Association (AWWA) and Water Pollution Control Federation (WPCF), Washington D.C.

Ashtaputre A.A., Shah A.K., (1995), Studies on the exopolysaccharide from *Sphingomonas paucimobilis* GS 1: Nutritional requirements and precursors forming enzymes, *Current Microbiology*, **31**, 234-238.

Belal E.B., (2013), Production of Poly-β-Hydroxybutyric Acid (PHB) by *Rhizobium melti* and *Pseudomonas stutzeri*, *Current Research Journal of Biological Sciences*, **5**, 273-284.

Ben Rebah F., Tyagi R.D., Prévost D., (2001), Acid and alkaline treatments for enhancing the growth of rhizobia in sludge, *Canadian Journal of Microbiology*, 47, 467.

Ben Rebah F., Tyagi R.D., Prévost D., Belbahri L., (2009), Poly-β-hydroxybutyrate production by fast-growing Rhizobia cultivated in sludge and in industrial wastewater, *Applied Biochemistry and Biotechnology*, **158**, 155-163.

Ben Rebah F., Tyagi R.D., Prévost D., Surampalli R.Y., (2002), Wastewater sludge as a new medium for Rhizobial growth, *Water Quality Research Journal of Canada*, **37**, 353-370.

Bonartseva G.A., Myshkina V.L., Zagreba E.D., (1994), Poly-ß-hydroxybutyrate content in cells of various Rhizobium species during growth with different carbon and nitrogen sources, *Microbiology*, **63**, 45-48.

Breedveld M.W., Miller K.J., (1998), *Cell-Surface Â-Glucans*, In: *The Rhizobiaceae*, Spaink H.P., Kondorosi A., Hooykaas J.J. (Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, 81-96.

Burton J.C., (1979), *Rhizobium Species*, In: *Microbial Technology*, Peppler H.J. (Ed.), Academic Press, Inc., New-York, 29-58.

Castellane T.C.L., Persona M.R., Campanharo J.C., Lemos E.G.M., (2015), Production of exopolysaccharide from rhizobia with potential biotechnological and bioremediation applications, *International Journal of Biological Macromolecules*, **74**, 515-522.

Cerning J., (1995), Production of exopolysaccharides by lactic acid bacteria and dairy propioni bacteria, *Lait*, **75**, 463-472.

Damery J.T., Alexander M., (1969), Physiological differences between effective and ineffective strains of Rhizobium, *Soil Science*, **108**, 209-216.

Donot F., Fontana A., Baccou J.C., Schorr-Galindo S., (2012), Microbial exopolysaccharides: main examples of synthesis, excretion, genetics and extraction, *Carbohydrate Polymers*, **87**, 951-962.

Duta P.F., Pesson de Franca F., Almedia Lopes L.M., (2006), Optimization of culture conditions for exopolysaccharides production in Rhizobium sp. using surface response method, *Electronic Journal of Biotechnology*, **9**, 391-399.

Fialho A., Martins L., Donval M., Leitao J., Ridout M., Jay A., Morris V., Sa-Correia I., (1999), Structures and properties of gellan polymers produced by Sphyingomonas paucimobilis ATCC 31461 from lactose compared with those produced from glucose and from cheese whey, *Applied and Environmental Microbiology*, **65**, 2485-2491.

Fouchet P., Jan S., Courtois J., Courtois B., Frelat G., Barbotin J.N., (1995), Quantitative single-cell detection of poly(β-hydroxybutyrate) accumulation in *Rhizobium meliloti* by flow cytometry, *FEMS Microbiology Letters*, **126**, 31-35.

Fraysse N., Couderc F., Poinsot V., (2003), Surface polysaccharide involvement in establishing the rhizobium–legume symbiosis, *European Journal of Biochemistry*, **270**, 1365-1380.

Gamar-Nourani, L., Blondeau, K., Simonet, J.-M., (1998), Influence of culture conditions on exopolysaccharide production by *Lactobacillus rhamnosus* strain C83. *Journal of Applied Microbiology*, **85**, 664-672.

Giavasis I., Harvey L.M., McNeil B., (2000), *Gellan gum*, *Critical Reviews in Biotechnology*, **20**,177-211.

Gonzalez J.E., Reuhs L., Walker G.C., (1996), Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*, *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 8636-8641.

Grothe E., Moo-Young M., Chisti Y., (1999), Fermentation optimization for the production of poly(ß-hydroxybutyric acid) microbial thermoplastic, *Enzyme and Microbial Technology*, **25**, 132-141.

Janczarek M., (2011), Environmental signals and regulatory pathways that influence exopolysaccharide production in Rhizobia, *International Journal of Molecular Sciences*, **12**, 7898-7933.

Kumari B.S., Ram R.M., Mallaiah K.V., (2009), Studies on exopolysaccharide and indole acetic acid production by *Rhizobium* strains from Indigofera, *African Journal of Microbiology Research*, **3**, 10-14.

Law J.H., Slepecky A.R., (1960), Assay of poly-β-hydroxybutyric acid, *Journal of Biotechnology*, **82**, 33-36.

Liu F., Li W., Ridgway D., Gu T., (1998), Production of poly-b-hydroxybutyrate on molasses by recombinant *Escherichia coli*, *Biotechnology Letters*, **20**, 345-348.

Meade J., Higgins P., O'Gara F., (1985), Production and storage of *Rhizobium legominosarum* cell concentrates for use as inoculants, *Journal of Applied Bacteriology*, **58**, 517-524.

Myers R.H., Montgomery D.C., (1995), *Response Surface Methodology, Process and Product Optimization using Designed Experiments*, John Wiley & Sons, Inc. New York.

Nampoothiri K.M., Singhania R.R., Sabarinath C., Pandey A., (2003), Fermentative production of gellan using Sphingomonas paucimobilis, *Process Biochemistry*, **38**, 1513-1519.

Pollock J.T., (2002), S*phingan Group of Exopolysaccharides (EPS)*, In: *Biopolymer*, Vol. 5, Vandamme E.J., Beats D.S., Stain-Buchel A. (Eds.), Wiley-VCH Verlag, Weinheim, Germany, 239-257.

Rinaudi L.V., González J.E., (2009), The low-molecular-weight fraction of exopolysaccharide II from *Sinorhizobium meliloti* is a crucial determinant of biofilm formation, *Journal of Bacteriology*, **191**, 7216-7224.

Singh A., Sharma P.K., Malviya R., (2011), Eco friendly pharmaceutical packaging material, *World Applied Sciences Journal*, **14**, 1703-1716.

Skinner F.A., Roughley R.J., Chandler M.R., (1977), Viability and cell distortion in *Rhizobium spp.*, *Journal of Applied Bacteriology*, **43**, 287-297.

Stam H., Van Verseveld H.W., de Vries W., Stouthamer A.H., (1986), Utilization of poly-β-hydroxybutyrate in free-living cultures of *Rhizobium* ORS571, *FEMS Microbiology Letters*, **35**, 215-220.

Sutherland I.W., (1985), Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides, *Annual Review of Microbiology*, **39**, 243-270.

Sutherland I.W., (1990), *Biotechnology of Microbial Exopolysaccarides*, Cambridge University Press, Cambridge, UK.

Tavernier M.L., Delattre C., Petit E., Michaud P., (2008), β-(1,4)-polyglucuronic acids – An overview, *Open Biotechnology Journal*, **2**, 73-86.

Tavernier P., Portais J.C., Saucedo J.E.N., Courtois J., Courtois B., Barbotin J.N., (1997), Exopolysaccharide and polyhydroxybutyrate coproduction in two *Rhizobium meliloti* strains, *Applied Environmental Microbiology*, **63**, 21-26.

Villano M., Valentino F., Barbetta A., Martino L., Scandola M., Majone M., (2014), Polyhydroxyalkanoates production with mixed microbial cultures, from culture selection to polymer recovery in a high-rate continuous process, *New Biotechnology*, **31**, 289-296.

Wang K., Wang Y., Zhang R., Li Q., Shen C., (2012), Preparation and characterization of microbial biodegradable poly(3-hydroxybutyrate-co-4-hydroxybutyrate)/organoclay nanocomposites, *Polymer Composites*, **33**, 838–842.

Xie F., Williams A., Edwards A., Downie J.A., (2012), A plant arabinogalactan-like glycoprotein promotes a novel type of polar surface attachment by *Rhizobium leguminosarum*, *International Society for Molecular Plant-Microbe Interactions*, **25**, 250-258.

Yan S., Tyagi R. D., Surampalli R. Y., (2006), Polyhydroxyalkanoates (PHA) production using wastewater as carbon source and activated sludge as microorganisms, *Water Science and Technology*, **53**, 175-180.