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RESEARCH ARTICLE

STATISTICAL APPROACH TO ASSESS THE PALM OIL MILL EFFLUENTS BIOTREATMENT WITH *YARROWIA LIPOLYTICA*

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Abstract: The present study reports the biotechnological conditions of the biodegradation process of Palm Oil Mill Effluent (POME) by using *Yarrowia lipolytica* MIUG D96 selected strain. For this purpose, a sequential statistical methodology, the Plackett-Burman Design (PBD) was applied for the selection of the biotechnological parameters that influence the process, aiming at enhancing the POME biodegradation. Among the tested parameters, the POME concentration, temperature, time, agitation rate, and inoculum concentration were identified as the most significant variables that influence the biodegradation process.

Keywords: POME, Plackett-Burman design, *Yarrowia lipolytica* MIUG D96, biodegradation.

Introduction

The palm oil industry is one of the major agro-industries in the Republic of Côte d'Ivoire, West Africa. The production of palm oil generates large quantities of polluted wastewater commonly referred to as palm oil mill effluent (POME). The most significant pollutant from palm oil mills is POME (Poh and Chong, 2009). Typically, one ton of crude palm oil production requires 5.0–7.5 t of water, over 50% of which ends up as POME. This wastewater is a viscous, brownish liquid

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containing about 95–96% water, 0.6–0.7% oil and 4–5% total solids (including 2–4% Suspended Solids, mainly debris from the fruit). POME is acidic (pH 4.0–5.0), hot (80–90 °C), nontoxic (no chemicals are added during oil extraction), has high organic content (Chemical Oxygen Demand 50,000 mg/L; Biochemical Oxygen Demand 25,000 mg/L) and contains appreciable amounts of plant nutrients (Borja and Banks, 1996; Singh *et al.*, 1999). POME contains about 4,000 – 6,000 mg/L of oil and grease (Ahmad *et al.*, 2005). The composition of POME mainly comprises of water, oil, suspended solids, dissolved solids and sand, total suspended solids (TSS), as well as cellulose wastes, vegetative matter, and colloidal slurry of water and solids. The suspended solids in POME which are the cellulolytic materials derived from palm mesocarp are considered important organic matter (Chin *et al.*, 1996) and constitute about 50% of the POME. Treatment and disposal of oily wastewater, such as POME, is currently one serious environmental concern. Palm oil mill wastes have existed for years but their effects on the environment are now more noticeable. The oily waste must be removed to prevent intermediate in water treatment units, avoid problems in the biological treatment stages, and comply with water-discharge requirements (Ahmad *et al.*, 2005). POME is an important source of inland water pollution when released into local rivers or lakes without treatment. POME contains lignocellulosic wastes with a mixture of carbohydrates and oil (Oswald *et al.*, 2002). Recently, various physical and chemical treatment processes have been designed to treat POME, however, the problem of chemical residues and total suspended solids (TSS) which are still present after the treatments has to be solved using more sustainable practices (Abdul Karim *et al.*, 2011). The use of microorganisms for the biological treatment of POME evaluated in this present study offers an alternative solution to reduce the TSS and organic content of the effluent. Palm oil industries are facing tremendous challenges to meet the increasing environmental regulations (Najafpour et *al.*, 2006). Thus, it is obvious that the presence of high levels of fat, oil and grease in the wastewater induces serious problems not only to the receiving water but also to treatment plants and waste collecting systems. Although oil is not generally recognized as a material that is discharged into Land Rivers, it can be a pollutant of these waters; not only as tin-coloured films but also in sufficient volumes to necessitate the closing of abstraction points (El-Bestaway *et al.*, 2005). It is therefore essential that the potential risks and effects of oil pollution are correctly evaluated. The various effluent treatment schemes that are currently used by the palm oil industry are listed in descending order: (a) anaerobic/facultative ponds (Wong, 1980; Rahim and Raj, 1982; Chan and Chooi, 1982), (b) tank digestion and

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mechanical aeration, (c) tank digestion and facultative ponds, (d) decanter and facultative ponds, and (e) physicochemical and biological treatment (Andreasen, 1982). The current method adapted for the treatment of palm oil mill effluent (POME) in most of the mills is the ponding system for about 85% of the mill's practice (Poh and Chong, 2009). This is not very effective in treating the pollutants in the POME to the stringent standards required (Jameel and Olanrewaju, 2011). The status and concentration of the oily matter/oil residue (oil and grease) after the treatment process is neglected and this suggests that the approach employed is not sustainable to minimize the environmental impact of oil and grease in POME. Moreover, the concentration range of oil and grease in POME is relatively higher than those obtained in toxic wastewater (Jameel and Olanrewaju, 2011). Thus, there is an imperious need for an effective treatment process for POME. The anaerobic digestion treatment of POME using various types of bioreactors by researchers and the ponding system in the mills uses unidentified microbial populations (McHugh *et al.*, 2003) to reduce the polluting power of wastes and wastewater. This involves a consortium of undefined microbial catalysers that must be able to support a complex series of biochemical reactions that mineralize the organic matter producing methane and carbon dioxide. These microorganisms are not established and hence the substrate they degrade and utilize is not ascertained. This led to poor effluent discharge into the environment as the performance of the microorganisms with regards to the reduction and removal rate of oily waste cannot be monitored since the microbial populations are not known.

Microbial degradation of oil wastewater has been a concern in recent years. A variety of microorganisms such as bacteria, moulds, and yeasts are capable of completely degrade oil wastewater (Erguder *et al.*, 2000, Kissi *et al.*, 2001, Ettayebi *et al.*, 2003, Dhouib *et al.*, 2006). Therefore, using microorganisms for treatment and bioremediation purposes affords a very efficient tool for purifying contaminated effluents and natural water (Glazer and Nikaido 1995). Using bacterial strains that possess high efficiency in accumulating toxic contaminants or biodegradation of persistent biodegradable matter has the potential to be used in treatment systems to remove pollution such as oil and grease or heavy metals from any polluted aquatic effluent (Campere *et al.*, 1993).

The main aim of the present study was to evaluate the POME biodegradation potential of a *Yarrowia lipolytica* selected strain by cultivation on the fermentation medium containing palm

waste and evaluation of the removal efficiency by using Plackett-Burman Design (PBD) to identify the biotechnological parameters with positive influence on the process.

Materials and Methods

POME samples and the yeast strains

The POME samples were collected from the site of a palm oil mill industry (ADAM AFRIQUE, Republic of Côte d'Ivoire (West Africa) in a sterile container. Two types of samples have been collected and coded MDG and RJ. The physicochemical characteristics such as BOD_5, total suspended solids (TSS), pH, conductivity (CON), salinity (SAL), total dissolved solid (TDS), acidity index (AI), and unsaturation index (UI) of the samples were determined in accordance with the methods published by American Public Health Association (APHA, 1995). Five yeast strains were used: two from Côte d'Ivoire (*Yarrowia lipolytica* CBS 6303 and *Yarrowia lipolytica* LGx64), two from Collection of Microorganisms (acronym MIUG) of Bioaliment Research Platform from the Faculty of Food Science and Engineering, "Dunărea de Jos" University, Galați, Romania (*Yarrowia lipolytica* MIUG D96 and *Yarrowia lipolytica* MIUG D6), and *Yarrowia lipolytica* ATCC 18942 which was purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA.

Inoculum preparation

The yeast strains have been first activated by cultivation on Potato Dextrose Agar (PDA) medium (Sigma Aldrich, Germany). Then the cells were transferred to a liquid medium (Yeast Extract Peptone Dextrose (YPD), and cultivated on an orbital shaker (Medline SI-300R, UK) for 48 h, at 30°C and 150 rpm. After cultivation, the biomass has been separated by centrifugation at 6000 rpm, 20 min at 4°C using the Hettich 320 R (Germany). The biomass obtained was washed with sterile saline solution (0.9%) before using it to inoculate the POME medium.

Selecting the yeast strains with the potential to transform the oil waste

Qualitative screening of the yeast strains

First, Spirit Blue Agar medium (Fluka, UK) supplemented with 5% of POME samples, coded MDG and RJ, was prepared and autoclaved at 121°C, for 15 min using the Panasonic MLS-3781L (Japan). The POME sterile media were transferred in sterile Petri plates and after solidification were inoculated "in point" with the selected yeast strains. The cultivation took place in stationary

conditions, for 48 h, at 30°C (Cotârleț *et al.*, 2020). After that, the diameter of the oil biotransformation zone (clear area) has been measured and expressed in millimeters (mm). At the end of this stage, two strains coded *Yarrowia lipolytica* CBS 6303 and *Yarrowia lipolytica* MIUG D96 were selected for further studies.

Quantitative screening of the yeast strains

Secondly**,** liquid medium supplemented with 5% POME, coded MDG and RJ, was autoclaved and then inoculated with $1x10^7$ CFU/mL inoculum of yeasts, dimensioned with Thoma counting chamber, and cultivated on orbital agitation for 120 h, at 30°C and 150 rpm. After cultivation, the yeast biomass was separated by centrifugation at 7,000 rpm for 10 min at 4°C and after that, for supernatant, the physio chemical parameters for oil biotransformation were assessed. The pH, BOD_5, acidity index (AI) and unsaturation index (UI), TDS, salinity (SAL) and conductivity (CON) are the parameters that have been taken into account as responses reflecting the biodegradation process.

Finally, the POME substrate, coded MGD, and the strain *Yarrowia lipolytica* MIUG D96 were selected to carry out the biodegradation process.

Screening of the parameters that influence the bioremediation process

Plackett-Burman Design

The biodegradation medium composition and the biotechnological conditions were optimized based on mathematical modelling and statistical analysis, according to the Plackett-Burman technique. The POME concentration, temperature, time, agitation, inoculum concentration and pH were considered as possible parameters (independent variables) that can affect the POME biodegradation. The total dissolved solid, conductivity, BOD_5 and salinity were considered as the analysed responses. The experimental matrix used for the design and level of variation of the independent variables are presented in Table 1. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). A first-order polynomial model (Eq. 1) was used for the mathematical modelling:

$$Y = \beta_0 + \Sigma\beta_i\chi_i \qquad (1)$$

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Where, *Y* is the predicted response, β_0 is the model intercept, β_i is the linear coefficient and χ_i is the level of the independent variable (Sawale and Lele, 2010).

Table 1. The PB Design independent variables and the levels of variation

Independent Variable	Units	Abbr.	Levels of variation	
			-1	+1
POME concentration	%	A	5	10
Temperature	°C	B	25	37
Time	h	C	120	240
Agitation	rpm	D	150	200
Inoculum concentration	CFU/mL	E	10^6	10^7
pH		F	4	6

Statistical analysis

Each experiment was carried out in triplicate and the data represents the mean of replicates. For the statistical analysis and mathematical modelling, Plackett-Burman experimental Design was employed by using Minitab 17 Statistical Software (version 1.0, LLC, Pennsylvania State University, USA) (Plackett and Burman, 1934). The model's analysis of variance was performed through Factorial regression. The model is statistically validated statistically where $p < 0.05$ and it is considered significant.

Results and discussion

Palm oil mill effluent physicochemical analysis

The physicochemical parameters of the POME samples, coded MDG and RJ, are shown in Table 2. Thus, the pH ranged from 4.39±0.20 to 4.73±0.25. The values of conductivity, salinity, TDS and TSS ranged from 8.55±0.25 to 9.68±0.75 mS/cm, 5.7±0.54 to 6.9±0.52, respectively 5.95±0.65 to 6.76±0.37 g/L and 52.7±0.77 to 56.8±0.69 g/L. The biochemical oxygen demand was between 628±22 and 999±15 mg O_2/L. The acidity index had values between 12.73±0.55 to 18.31±0.18 and finally, the unsaturation index varied from 31.13±0.67 to 46±0.89.

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Table 2. Physicochemical characteristics of POME

Parameters	Values	
	MGD	**RJ**
pH	4.39±0.20[a]	4.73±0.25[a]
Conductivity (mS/cm)	8.55±0.25[a]	9.68±0.75[a]
Salinity	6.9±0.52[a]	5.7±0.54[a]
TDS (g/L)	6.76±0.37[a]	5.95±0.65[a]
BOD_5 (mg O_2/L)	999±15[a]	628±22[b]
TSS (g/L)	52.7±0.77[a]	56.8±0.69[a]
Acidity Index (AI) (mg NaOH/g)	18.31±0.18[a]	12.73±0.55[b]
Unsaturated Index (UI) (g/I_2/100 g)	46±0.89[a]	31.13±0.67[b]

Online, values with the same letter are not statistically different (p>0.05).

The pH results obtained show the acid character of the POME as indicated by certain authors. Salihu *et al.* (2010) stated that POME had an acidic pH between 4.0 and 5.0. It is therefore necessary to treat it before it is (the POME) released into the environment. Conductivity, salinity, TDS and TSS have very high values. POME would be very polluting, heavily loaded with organic matter and particularly affect the quality of the waters into which it is discharged (receiving environments). The colour of these wastes and their high organic load lead to high oxygen consumption leading to eutrophication of the waters (Benyahia and Zein, 2003). The organic fraction contains macromolecules, such as proteins (11.1%), polysaccharides (50.5%), and lipids (12%) including triglycerides composed of unsaturated and saturated fatty acids. It also contains (Palm Oil Mill Effluent) significant amounts of minerals 80% of which are soluble (Mg, K, N, P) and 20% insoluble (Ca, Fe, Zn, Cu) (Iwara *et al.*, 2011). BOD_5 is an indicator of the activity of microorganisms with respect to organic matter. The values obtained are much lower than those of some authors. Thus, according to Hassan-Aboushiba *et al.* (2011), POME consists of an average of 25 g O_2/L. This difference could be explained by the palm oil extraction process. The unsaturation and acid indices are indicators of the acidity and unsaturation of POME. Indeed, POME is made up of a majority of 51% saturated and 49% unsaturated fatty acids.

Qualitative screening of the yeast strains with the potential to transform the oil waste

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The strains of *Yarrowia lipolytica* were inoculated on Spirit Blue agar supplemented with POME. Table 3 presents the measurements of the clear areas, which varied depending on the substrates from 22±0.54 to 25±0.35 mm, for *Yarrowia lipolytica* LGx64; 35±0.24 to 43±0.22 mm, for *Yarrowia lipolytica* CBS 6303; 44±0.68 to 50±0.65 mm, for *Yarrowia lipolytica* MIUG D96; 29±0.37 to 43±0.48 mm, for *Yarrowia lipolytica* MIUG D6 and 27±0.28 to 28±0.67 mm, for *Yarrowia lipolytica* ATCC.

Table 3. POME biotransformation potential by yeasts cultivation on Spirit Blue agar

Strains	**Biotransformation potential, mm**	
	POME coded MGD	**POME coded RJ**
Yarrowia lipolitica CBS 6303	43±0.22^{a}	35±0.24^{a}
Yarrowia lipolytica LGx64	25±0.35^{b}	22±0.54^{b}
Yarrowia lipolytica MIUG D96	50±0.65^{c}	44±0.68^{c}
Yarrowia lipolytica MIUG D6	43±0.48^{a}	29±0.37^{d}
Yarrowia Lipolytica ATCC 18942	28±0.67^{b}	27±0.28^{d}

In column, values with the same letter are not statistically different ($p>0.05$).

Yarrowia lipolytica strains coded, MIUG D96 and CBS 6303, were therefore chosen, for further experiments, for their ability to metabolize POME having the higher diameter of the biotransformation areas. In addition, by using the coded MGD of POME, it was observed an improved biotransformation potential. The POME, coded RJ, had a low-fat content, which could explain the lower biotransformation potential of the tested yeasts. Indeed, the MGD samples were collected directly at the outlet of the mill valves, while RJ was collected after a lagoon system.

Yarrowia lipolytica effectively degrades hydrophobic substrates and can therefore be successfully used to purify palm oil mill effluent (POME) and olive mill wastewater (OMW) (Bankar *et al.*, 2009). OMW is the wastewater remaining after the olive oil pressing process (Gonçalves *et al.*, 2009) containing sugars, polyalcohols, polyphenols, tannins, lipids and pectins. These compounds cause high chemical oxygen demand (COD) of waste (Papanikolaou *et al.*, 2008). Oleaginous yeast species (*Y. lipolytica*) produced biomass and other products such as organic acids or enzymes by consuming organic components from substrates with OMW (Papanikolaou *et al.*, 2008; Gonçalves *et al.*, 2009). Lanciotti *et al.* (2005) analysed the potential of different strains of *Y. lipolytica* for growth in OMW media and the ability of yeast to reduce COD values. Depending on

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the composition of the culture medium, it was possible to synthesize enzymes with different specificities.

Quantitative screening of the yeast strains with bioremediation potential

A fermentative medium based on POME has been sterilized and inoculated with $1x10^7$ CFU/mL inoculum of yeasts, and cultivated on an orbital shaker for 120 h, at 30°C and 150 rpm. After cultivation, the biomass was separated by centrifugation and the physiochemical parameters for oil's bioremediation were assessed. Table 4 presents the values of parameters during the biodegradation achieved by the two strains in the presence of the two POME samples. It emerges that *Y. lipolytica* MIUG D96 allowed a greater reduction of these parameters in both MGD and RJ, unlike *Y. lipolytica* CBS 6303. Thus, the BOD_5 was from 514±13 to 728±20 mg O2/L, the pH from 4.62±0.15 to 5.61±0.42 and the unsaturation index from 16.16±0.67 to 24.18±0.94 g/I_2/100g when *Y. lipolytica* CBS 6303 was cultivated in the samples while BOD_5 recorded values from 454±12 to 650±24 mg O_2/L, pH from 4.62±0.22 to 8.04±0.56 and the unsaturation index from 5.10±0.35 to 22.26±0.74 g/I_2/100g for *Yarrowia lipolytica* MIUG D96.

Table 4. Selection of the yeast strain based on the bioremediation properties

Strains	**POME**							
	MGD				**RJ**			
	pH	BOD_5, mg O_2/L	AI, mg NaOH/g	UI, g/I_2/100 g	pH	BOD_5	AI, mg NaOH/g	UI g/I_2/100 g
Y. lipolytica CBS 6303	4.62±0.15[a]	514±13[a]	4.62±0.75[a]	16.16±0.67[a]	5.61±0.42[a]	728±20[a]	13.79±0.16[a]	24.18±0.94[a]
Y. lipolytica MIUG D96	4.62±0.22[a]	454±12[b]	7.37±086[b]	5.10±0.35[b]	8.04±0.56[b]	650±24[b]	12.98±0.34[a]	22.26±0.74[a]

In column, values with the same letter are not statistically different (p>0.05).

At the end of the selection process, *Yarrowia lipolytica* strain coded, MIUG D96, was chosen, for optimization experiments, by using the palm oil mill effluent, coded MGD.

Plackett-Burman Design for selection of the variables that influence the biotransformation process

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Table 5 presents the results of the correlative effects of the analysed parameters using the PB Design. The regression analysis was performed on the results and a first-order polynomial equation was determined. Statistical analysis of the analysed response is presented in Table 6. The p-values lower than 0.05 denoted that the model terms and the model are significant. For the acidity index, the model was validated statistically (p = 0.021), but the factor pH was eliminated. The important factors are POME concentration, % (p = 0.007) and agitation (p = 0.007). For the unsaturated index, the model was validated statistically (p = 0.044), but the factor pH was eliminated. For BOD_5, the model was validated statistically (p = 0.037). The important factors are temperature, °C (p = 0.009) and pH (p = 0.030). For TDS, the model is statistically validated (p = 0.001). The important factors were POME concentration, (p = 0.001), inoculum concentration (p = 0.000) and the pH (p = 0.037). For SAL, the model was validated statistically (p = 0.001). The important factors were POME concentration, (p = 0.001), temperature, (p = 0.019) and inoculum concentration, (p = 0.000). For conductivity, the model is validated statistically (p = 0.001). The important factors were POME concentration, (p = 0.001), inoculum concentration, (p = 0.000) and the pH (p = 0.034).

The Pareto chart of the standardized effects is presented in Figure 1.

Several works have specified the interest of the various microorganisms including the yeast *Yarrowia lipolytica* in the treatment of aerobic biodegradation and detoxification (Alloue *et al*., 2005), particularly indicating *Y. lipolytica* as a potential agent for the purification of effluents and the reduction of pollution. The microbial load is related to the number of effluents, indeed, but more important is the correlation between the microbiome and the composition of the effluents to fulfil the microorganisms' nutritional requirements that will result in a performant bioremediation process. The development of a biodegradation model requires a careful choice of the nutrients, growth factors and parameters of the bioprocess. In this study, the experiments were conducted to evaluate the effect of the selected independent variables (biotechnological parameters), many of them contributing to the *Yarrovia lipolytica* metabolism during the biodegradation of POME. The most important factors concerning biodegradation are represented by POME concentration, temperature, time, agitation and inoculum concentration. The work of Oswald *et al*. (2002) also confirmed that POME biodegradation was influenced mostly by POME concentration, temperature, time, agitation rate and inoculum concentration. The TDS, and BOD_5, as well as the salinity, allowed the measurement of the biodegradability of organic and mineral matter. Lanka

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and Pydipalli (2018) also claimed that BOD_5 is an important response factor during POME biodegradation.

Table 5. The PB for the screening of the most significant independent variables that influence the biodegradation of the POME by *Yarrowia lipolytica*

Run	Independent variables						Responses					
	A*	B	C	D	E	F	AI	UI	BOD_5	TDS	SAL	CON
1	7.1	31	180	175	$5.5x10^6$	5	0	13	0	618	0.6	1039
2	5	25	240	200	$1.0x10^7$	4	0.1	8.8	0	725	0.6	1215
3	10	37	120	200	$1.0x10^6$	4	0.2	1.3	33.54	752	0.7	1261
4	5	25	120	150	$1.0x10^6$	4	0.2	1.7	1671	337	0.3	569
5	10	25	240	200	$1.0x10^6$	6	0.2	9.6	0	716	0.6	1210
6	7.5	31	180	175	$5.5x10^6$	5	0.1	3.2	0	605	0.5	1018
7	5	37	240	150	$1.0x10^7$	4	0.1	6.4	4006	850	0.8	1424
8	5	37	120	150	$1.0x10^6$	6	0.2	0	3015	521	0.5	873
9	10	37	120	200	$1.0x10^7$	4	0.1	2.7	2746	858	0.8	1438
10	5	25	120	200	$1.0x10^7$	6	0	2.3	0	782	0.7	1310
11	7.5	31	180	175	$5.5x10^6$	5	0.2	10.2	0	609	0.5	1023
12	5	37	240	200	$1.0x10^6$	6	0	3.8	0	538	0.5	905
13	10	37	240	150	$1.0x10^7$	6	0.3	15.9	0	1280	1.1	2130
14	10	25	120	150	$1.0x10^7$	6	0.3	2.7	0	992	0.9	1668
15	10	25	240	150	$1.0x10^6$	4	0.3	12.7	0	654	0.6	1098

*A, POME concentration, %; B, temperature, °C; time, h; D, agitation, rpm; E, inoculum concentration, CFU/mL; F, pH

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Table 6. Factorial regression of the model TDS, mg/L versus POME concentration (%), Temperature (°C), Time (h), Rpm, Inoculum concentration (CFU/mL), pH

Source	Sum of Squares	Degree of freedom	Mean square	F-value	p-Value
Model	650425	7	92918	17.28	0.001
A-POME concentration, %	187250	1	187250	34.82	0.001
B-Temperature, °C	29304	1	29304	5.45	0.052
C-Time, h	22620	1	22620	4.21	0.079
D-Agitation, rpm	5764	1	5764	1.07	0.335
E-Inoculum concentration, CFU/mL	323080	1	323080	60.08	0.000
F-pH	35534	1	35534	6.61	0.037
Error	37641	7	5377		
Total	688066	14			

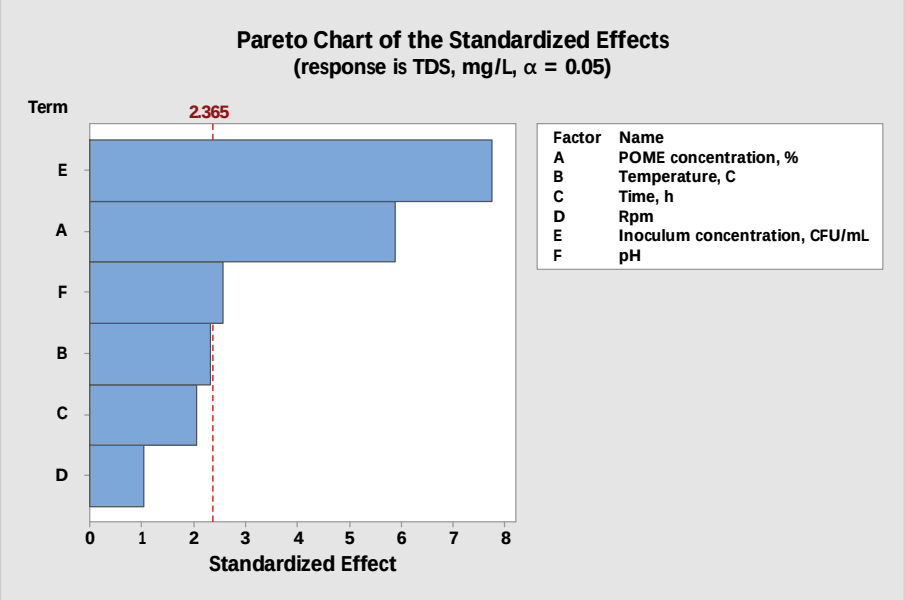

Figure 1: Pareto chart that highlight the impact of independent variables on TDS as response

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Conclusions

This study demonstrated the ability of *Yarrowia lipolytica* MIUG D96 strain to grow and metabolize POME, minimizing the environmental impact of this waste. Through mathematical modelling and statistical analysis, the fermentation conditions aiming at increasing the POME biodegradation using a selected strain of yeast were established. The total dissolved solids, conductivity, BOD_5, and salinity were considered as responses. The most important parameters with impact on POME biodegradation are represented by POME concentration, temperature, time, agitation rate, and inoculum concentration. These results are preliminary. Further, the optimization of the biodegradation process will be established by using the selected yeast strain through the Response Surface Methodology.

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