Published by :
http://www.ijert.org

International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 6 Issue 06, June - 2017

Mixture Experimental Design in the Development of a Bio Fertilizer from Fish Waste, Molasses and Scum

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***Abstract*— The objective of our study is to apply response surface methodology to the design and analysis of composite experiments established in order to optimize 15 days of biotransformation of ternary mixture of industrials wastes: fish waste, molasses and scum (derived from the sugar refining process through juice carbonatation) in order to produce an interesting biofertilizer with a best quality. To study a better formula insuring favorable biotransformation by changing proportions of the ternary mixture, an experimental design is utilized consisting of 7-point simplex centroid designs with constrained regions. The fitted model provides information needed to predict optimum formulation, i.e. richness in phosphorus, nitrogen and good hygiene parameters.**

The results show that the formulation including early 68% of fish waste, 13% of molasses and 19% of scum is the best. It was stable from the fifth day, with a stable pH, rich in both, phosphorus, nitrogen and is hygienic. The performance tests as a biofertilizer have shown that our product is more interesting than some commercially available products.

Keywords*— Mixture model, Biofertilizer, Biotransformation, Fish waste, Scum, Experimental design.*

I. INTRODUCTION

The agrifood industry generates many organic wastes. As it is mismanaged, the impact can be very significant and will raise economic and ecologic problems. Last years, fish consumption has been on the rise. Such a trend has resulted in the generation of large amounts of fish waste, mostly from industrial processes, which have not been utilized efficiently, as untreated fish waste is customarily disposed of via landfill, incineration, or by dumping into the sea.

These amounts could be recycled as a potential source of phosphorus and nitrogen in biofertilizers in agriculture. Conventional methods for reutilization of fish waste include ensilation [1], and production of high-protein animal feeds [1,2]. Composting and biotransformation has also been suggested as a viable solution, and a way of producing agricultural soil amendment [3]. Brewer's yeast and leaven (yeasts and lactic acid bacteria) are using into the biotransformation of fish waste and remove fish odor.

Which inhibits pathogenic bacteria, without any degradation of nutritional qualities of the products [3,4].

The technological biotransformation theory suggests that in order to get an interesting product, the elemental composition of starting mixtures (source of carbon, nitrogen, phosphorus) must be balanced and must be optimized and the conditions necessary for growth and microbial activity must be ensured. It is therefore essential to distinguish what optimal configuration that will ensure that this biotransformation is directed toward the generation of a product with high added value [5, 6].

The objective of this study is to use statistical approach to design and analysis of mixture experiments containing covariate(s) that will yield a better understanding and study biotransformation of ternary mixture of industrials wastes: fish waste, molasses and scum (waste from sugar industry) in order to produce an interesting biofertilizer with a best quality. This methodology was chosen for its simplicity and its opportunity to offer good information, reducing number of tests, time and cost incurred.

II. MATERIALS AND METHODS

A. Preparation of Mixture

The scum recovered from the sugar industry COSUMAR are dried, ground to a fine powder. It is then mixed with industrial waste of sardine (Sardinapilchardus), which essentially contain the bones, guts and heads, and have been also ground in an ice crusher. Molasses is also added. The Saccharomyces cerevisiae yeast is used as biotransformation agent and was added at a fixed mass ratio of 1%. The work presented here aims to recover fish waste by associating them with scum and molasses. Several fractions of such 3 components were studied with the aim of identifying better formula, allowing a favorable biotransformation to produce a mixture with interesting qualities, which can be used as fertilizer.

B. Chemical and physicochemical analysis

The pH was determined using a pH-meter (Fisher Scientific, Basic AB15). The dry matter (DM) was determined daily by oven drying of 3g at 60°C for 24 h, three times per day. Conductivity and temperature were measured daily by HANNA Instruments, EC215.Total nitrogen was determined according to the Kjeldahl method using sulfuric acid for the digestion of organic samples [7]. The rate of available phosphorus was determined by spectrophotometric assay based on the reaction

International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
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with ammonium molybdate according to the French standard NF EN 1189 T90-023 and Européenne (EN 1189: 1996) [8].

C. *Microbiological analysis*

Microbiological analysis performed in the first and the 15th day. A Columbia blood agar is prepared to determine the presence of *streptococcus* reflecting proteolytic effects [9]. The presence of *staphylococcus* (lipolytic marker) is determined at a mannitol salt agar [10]. A Mac Conkey agaris used to visualize the presence of *Escherichia coli* (hygiene indicator) [11].

D. *Data Analysis*

In this study, we were focus on the evolution of the quality of biotransformation mixture depending on the composition of the starting mixture and further optimization with respect to time. The ternary surface response diagram, polynomial model and principal component analysis (PCA) were generated by Statistica software® 10 (StatSoft, USA).These tools are used to identify factors that have a statistically significant influence on the nutrient quality of the mixtures.

To optimize these treatment parameters, the three independent variables used in this study were: fish waste, molasses and scum. The experimental design consists of 7-points in the ternary diagram with constrained regions (fish wastes > 50%, molasses> 12.5%) (Table 1, Fig.1).

TABLE 1 COMPOSITION OF INITIAL TESTS

Composition N°	Fish waste (%)	Molasses (%)	Scum (%)
1	50.00	50.00	0.00
2	62.50	25.00	12.50
3	87.50	12.50	0.00
4	50.00	12.50	37.50
5	68.75	12.50	18.75
6	68.75	31.25	0.00
7	50.00	31.25	18.75

Based on a previous study conducted by our laboratory, the pH values in the mixtures with molasses at 15-25% indicate good fermentation, whereas mixtures with low molasses (5% and 10%) become alkaline at the end of the 6th day. So the variability range of the molasses used must be better than 12.5% and the use of a fraction of fish less than 50% is not achievable [3].

Experimental data from different treatments were analyzed using PCA. Therefore, these various formulas are prepared and monitored for 15 days by quality control parameters: pH, dry matter, total nitrogen, phosphorus analyzes and microbiology.

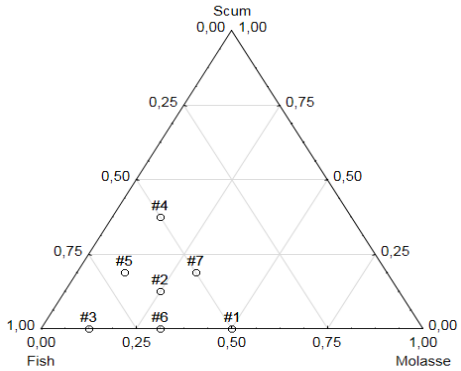

Fig 1. Simplex-centroid design with 7 points arrangement in the weight fraction ternary diagram with constrained regions.

E. *Seed germination test*

To evaluate the phytotoxicity of the mixture culture (which is based on fish waste) a seed germination test was carried out according to Kyun Kim (2010) [12]. Ten milliliters of culture was agitated for 10 min, filtered through a 0.45 mm membrane filter kept at 4°C until tested.

For tests of seed germination and root length, 5 mL of filtrate was pipetted into a sterile Petri dish lined with Whatman filter paper. Ten barley (*Hordeum vulgare*) seeds were evenly placed in each dish (three replicates for each sample) and the seeds were incubated at 25°C in the dark at 75% of humidity. DW was used as a control. Seed germination and root length in each plate were measured after 72 h. The percentages of relative seed germination (RSG), relative root growth (RRG) and germination index (GI) were calculated as the following formula [13]:

$$RSG\ \% = \frac{\text{Number of seeds germinated in extract} * 100}{\text{Number of seeds germinated in distilled water (DW)}}$$

$$RRG\ \% = \frac{\text{Mean radical length in extract} * 100}{\text{Mean radical length in distilled water (DW)}}$$

$$GI\ \% = \frac{RSG * RRG}{100}$$

F. *Chemical and physicochemical characterization of the soil*

Before the barley crop, a soil sample of each column was collected. The samples were air dried, sieved (<2 mm) and stored analysis. The pH of the soil was determined using a pH-meter (Fisher Scientific, Basic AB15) and the electrical conductivity was determined by HANNA Instruments, EC215. Total organic carbon was determined by Walkley-Black method [14].

The exchanged cations were determined by flame emission spectrophotometry (Na and K) (Digital Flame Photometer PFP7/C, JENWAY®). The compositions of total nitrogen and rate of phosphorus were determined by the methods described previously respectively [7, 8].

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G. Toxicity test and fertilization

The study of the toxicity of this product on plant germination was carried out on the *Hordeum vulgare* species of barley of the Amira variety that is marketed in Morocco and provided by the National Institute for Agricultural Research. The barley was grown in cases with a depth of 6 cm; the seeds were deposited at a depth of 3 cm and covered with soil.

The application rate was 0.054 g per 36 cm^2 of soil, 150 kg /hectare equivalent (the minimum- recommended by the FAO fertilizer use for cereal crops in Morocco dose) [15]. Plastic square plug trays measured 3 columns x 4 cases and each case measures 36 cm^2, were used for barley crop testing. Column 1 contained the control soil without amendment, column 2 contained commercial fertilizer (Algoflash), and column 3 contained natural product developed during this study, representing optimal physicochemical results.

III. RESULT AND DISCUSSION

A. Physical and textural properties of mixtures

At the end of the biotransformation process, all mixtures were characterized by a homogeneous appearance, dark color, lumpy structure and development of a pleasant odor (Fig.2). Physical and textural properties of biotransformation product are dependent on the process conditions such as biotransformation type, feed moisture and temperature [17].

Different studies have reported that the product characteristics such as color, texture, appearance and odor have an important bearing on the acceptability of the final product [3,4,16]. Even in the course of composting there is little emission of unpleasant odors. If such odors exist, they represent an incorrect evolution of composting (lack of oxygen) [18].

Fig 2 .Mixtures 5, 6 and 7 after 15 days of biotransformation

B. Temperature, pH and conductivity evolution

The study has shown that variations in the temperature are not more than 1°C. It can be considered as insignificant. This can be explained by the low thickness of the biotransformation mixture in the container as well as the regular mixing applied. All mixtures have a temperature which changes with the ambient temperature.

The biotransformation process applied in this study therefore allows good practice mastery of temperature.

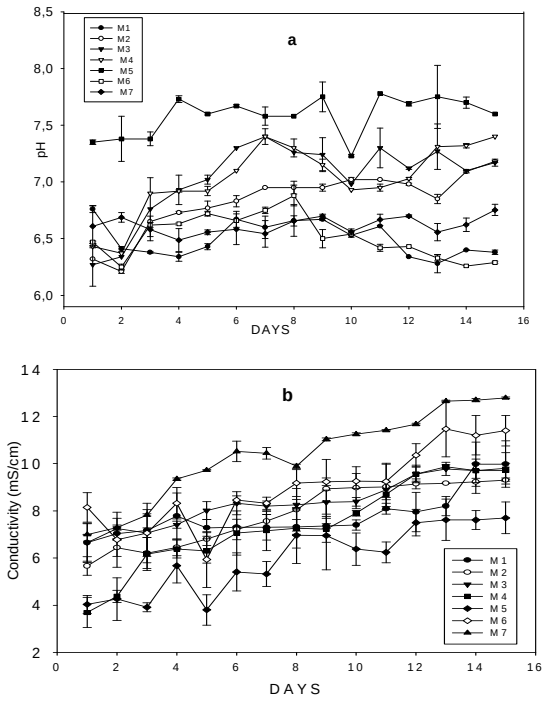

Fig 3. Evolution of pH (a) and Conductivity (b)

Based on the results of monitoring pH during 15 days (Fig. 3a), M1 and M6 compositions have shown pH values slightly acidic. The biotransformation of other mixtures has moderately neutral pH, about 7.5. The stabilization of pH in the testing of all compositions was due to the fermentative activity of the yeast, *Saccharomyces cerevisiae* [16]. The reported values since yeasts and bacteria involved in the biotransformation have their pH optimum between 5 and 8.5 [17].

The monitoring of pH indicated that after 5 days of testing all the compositions were mature. pH stabilization was due to the reduction of activity of microorganisms [18]. A pH marked by a slight acidity is a witness of a favorable biotransformation.

As for conductivity, we note that it increased slightly for all the mixtures for 15 days from a value of 3.69mS/cm, while M7 composition alone achieved an increase up to 12.79 mS/cm in the 15th day (Fig. 3b). All the mixtures were presented a rise in conductivity during the process of biotransformation. This evolution is inversely correlated with pH value; the more pH is away from neutrality the more the conductivity is high, probably due to increased ionic forms [1].

Generally, the presence of ions could be beneficial to plants, but it has been shown that too high values of conductivity threatens the survival of microorganisms and reduces the quality of the compost [1].Based on the work of Taiek et al. [2, 4], this may be due to the total degradation of carbohydrates by yeast and the release of volatile substances [16].

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C. *Phosphorus, total nitrogen and dry matter evolution*

Table 2 shows the evolution of the rate of phosphorus, total nitrogen and dry matter in different compositions.

TABLE 2.PHOSPHORUS, TOTAL NITROGEN AND DRY MATTER CONTROL OF BIOTRANSFORMED PRODUCT

Compositions	DM (%)	P(mg/100g)	N (g/100g)
1	43.33 ±0.00	41.06 ±0.00	2.60 ± 0.00
2	40.00 ± 0.01	45.77 ±0.00	2.70 ±0.02
3	33.33 ± 0.00	40.80 ±0.00	2.80 ± 0.10
4	56.70 ± 0.10	34.76 ±0.09	2.60 ±0.17
5	**60.00 ± 3.30**	**51.20 ±0.10**	**3.30 ±0.10**
6	**46.66 ± 3.34**	**56.84 ±0.02**	**1.80 ±0.70**
7	71.17 ± 2.00	18.52 ±0.17	3.66 ± 0.88

To test the nutritional parameters of biotransformed products made in the study, we realized a monitoring during 15 days. The results are shown in table 2. The dry matter of mixtures increases during the process. Based on the work of Taiek et al. [2, 4], the increase of the dry matter in other mixtures may be due to the loss of water by evaporation or by the loss of carbon dioxide and ethanol (by evaporation) during fermentation [4]. So the scum improves the rate of dry matter.

Concerning the rate of phosphorus, M5 and M7 compositions have a rate as high as the other compositions, surely, because they have more sardine wastes and that such fish is rich in terms of phosphorus [4]. Scum is a source of this element, known for their richness in minerals and phosphorus [19]. Therefore, the addition of yeast and molasses to the mixture thus helps in the conservation of phosphorus. A richer formula in fish waste and molasses allows phosphorus rates to be improved.

During processing, the total Kjeldahl nitrogen content marks stabilization for M1 composition. Unlike other tests, such rates increased during the 15 days of biotransformation. M5 and M7 had a remarkable evolution in the nitrogen content compared to the other mixtures. This is explained by the activity of microorganisms and the loss of volatile and liquid function [4]. We retain that the formulas richest in fish waste, scum and molasses are more enriched with nitrogen. As shown in the table 2, compositions 5 and 6 have the highest and lowest values, respectively, in terms of nitrogen, phosphorus and dry matter.

D. *Microbiological tests*

Table 3 presents the results of the bacteriological tests conducted on the first and last day, to identify the presence of strain indicator of hygiene and alteration. The product must have a number of staphylococcus and streptococcus that does not exceed ten Colony Forming Unit per gram of product (<10 CFU/g), and have less than one hundred CFU per gram (<100 CFU/g) for Escherichia coli [20].

TABLE 3 MICROBIOLOGICAL ANALYSIS

	M1		M2		M3		M4		M5		M6		M7	
Days	T 0	T f	T 0	T f	T 0	T f	T 0	T f	T 0	T f	T 0	T f	T 0	T f
Escherichia coli	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Staphylococcus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus	+	-	+	-	+	-	+	-	+	-	+	-	+	-

(++:> 100UFC; +:>10 CFU - : absence)

The microbiological test was also favorable. All tests revealed no presence of *E. coli*, *Staphylococcus*, and *Streptococcus* at the end of the fermentation process. Hygienisation, and inhibition of proteolysis and lipolysis in the mixtures containing yeast, due to its probiotic activity [3].

All these tests showed the importance of yeast in the making of a favorable biotransformation of fish waste, which inhibits pathogenic bacteria without any degradation of nutritional qualities of the product nor produces a toxic effect.

E. *Statistical analysis*

- Principal Component Analysis (PCA)

The following graphs illustrate the principal components analysis of the data (Fig. 4). We note that M5 (The richest in fish), is separated from the other mixtures because of its high content nitrogen and phosphorus. Similarly, M6 has a different quality from the others, because of its high content of phosphorus but low in nitrogen. Unlike M7 which represents the opposite. The other mixtures (1, 2, 3 and 4) are grouped together, which means that their properties are almost similar.

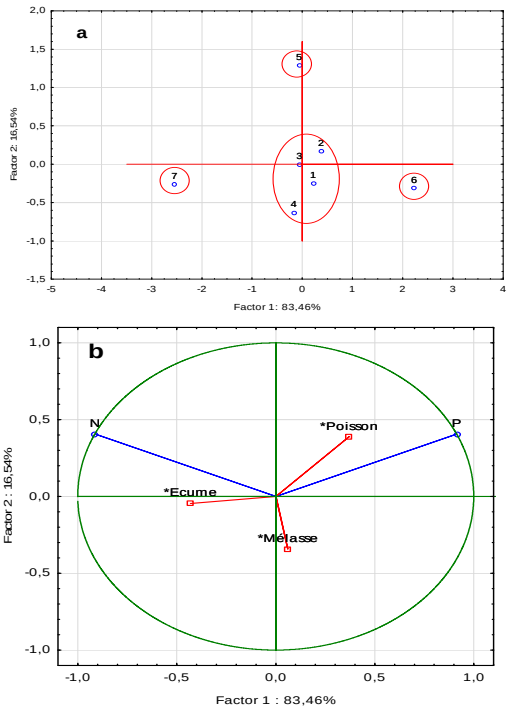

Fig 4. The Principal Component Analysis (PCA) in two dimensional patterns which explained almost 100% of the total variance. (a) The Biplot consisting projection on PC1 (83.46%) and PC2 (16.54%), (b) Correlation circle between principal components and original variables.

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- Response Surface Model estimates

The experimental design for the study of response surface is an important step; Very demanding in tests that's why we found important to considerate only the factors who's the response was previously verified [22].

This study tries to translate the potential of nitrogen, phosphorus and dry mater to biotransformation through the linear and quadratic model. The results obtained allowed the establishment of surface response diagrams. A summary of pertinent model and regression coefficient over each term in the model for quality parameters is given in Table 4.

TABLE 4 SUMMARY OF PERTINENT RESPONSE SURFACE MODELS FOR ELEMENTARY WEIGHT FRACTION IN THE MIXTURE.
F: weight fraction of fish waste, M: weight fraction of molasses, S: weight fraction of scum.

	Response surface model	Signifi -cance (α)	Adjust -ed R^2
N	Linear model N=+2.59*F+2.57*M+3.175*S	0.05	0.1407
	Quadratic model N=+2.82*F+2.62*M+2.62*S-4.02* F* M + 1.98 * F *S+3.82*M*S	0.05	**0.9726**
P	Linear model P=+52.29*F+39.42*M+32.13*S	0.05	0.2827
	Quadratic model P=+40.63*F+40.89*M+34.59*S+67.02*F*M+ 5.06 *F*S-74.18*M*S	0.05	**0.9959**
DM	Linear model DM=+36.514*F+48.982*M+65.014*S	0.05	0.4972
	Quadratic model DM=+34.98*F+44.98*M+58.35*S+29.6*F*M+ 26.92*F*S+51.60*M*S	0.05	**0.6494**

R^2=1-{[(N-1)/(N-p)][SSE/(SSE+SSR)]}; SSR, regression sum of square; SSE, residual sum of squares.

The postulated models were chosen based on the adequate lack of fit with significant confidence level 95% and the analysis of variance with satisfactory values of R^2. These values close to 1 testify to the good quality of our models.

Figs. 5, 6 and 7, show the response surface diagrams with changes in nitrogen, phosphorus and dry matter respectively. According to figures bellow, we notice that, the increasing of sardine wastes and scum encourages an increase in phosphorus and nitrogen levels, respectively.

The biotransformation is better when the mixture is rich on fish. The results show that quadratic model is the most pertinent. Combining between these figures, the area of interest between mixture number 2 and number 5 can be delimited in the ternary diagram. This one, represent optimal content of both the nitrogen, phosphorus and dry matter.

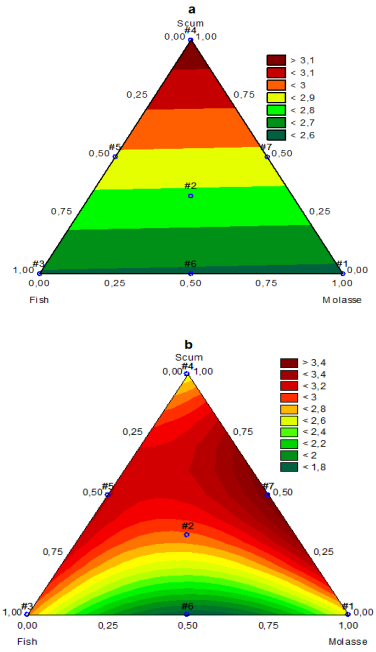

Fig 5. Ternary response surface diagrams with changes in nitrogen concentration calculated from the fitted model equations listed in Table4 (a) Linear model (b) Quadratic model.

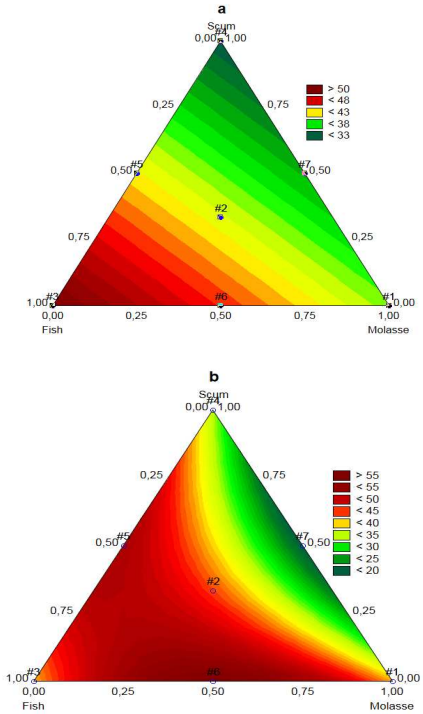

Fig 6. Ternary response surface diagrams with changes in phosphorus concentration calculated from the fitted model equations listed in Table 4 (a) Linear model (b) Quadratic model.

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ISSN: 2278-0181
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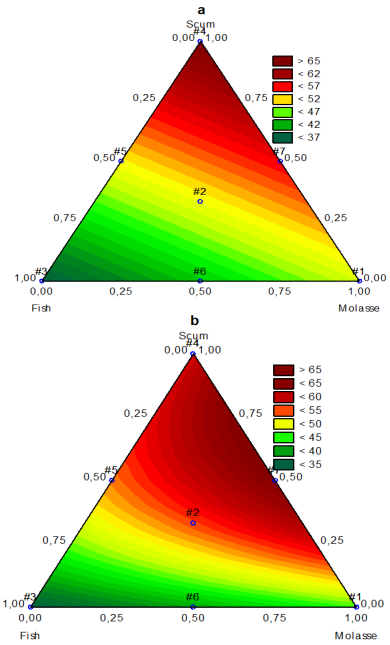

Fig 7. Ternary response surface diagrams with changes in dry matter concentration calculated from the fitted model equations listed in Table 4 (a) Linear model (b) Quadratic model.

F. Seed germination test

Since the product (M5) contains compounds potentially useful for plants, an attractive application is its use as a fertilizer; however, its application depends on the absence of any toxicity. Phytotoxicity was assayed on the 72 h at the same dilution and compared with that of a commercial fertilizer. The results of germination test on barley shows that M5 had no inhibitory effect on the germination.

The GI value reached approximately about 70 % for M5 and 55 % for commercial fertilizer (Fig. 8). M5 has a better GI value, implying feasible development of a biofertilizer from fish wastes, scum and molasses.

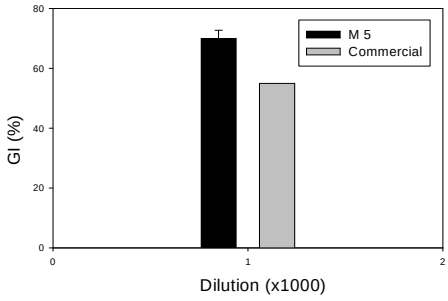

Fig 8.Percentages of germination index (GI) for biotransformed of ternary mixture (M5) at 1000 fold dilutions.

G. Characterization of the soil, commercial fertilizer and fertilization tests

Table 5 shows the results of chemical and physicochemical properties of the experimental soil and commercial fertilizer.

TABLE 5a CHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF THE EXPERIMENTAL SOIL

Parameter	Value
pH	6.07 ± 0.70
N (%)	0.64± 0.00
P (%)	0.14 ± 0.80
TOC (%)	6.46 ± 0.31
K (%)	0.7 ± 1.20
Na (%)	0.35 ± 0.01
EC (mS/cm)	1.08± 0.05

TABLE 5b CHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF THE COMMERCIAL FERTILIZER

Parameter	Value
pH	6.7
P (%)	6
K (%)	8
N (%)	12

The values obtained allow us to deduce that the soil can be considered as a good quality which can be used to test the biofertilizer (M5) in the barley crop [21]. It is very important to improve the utilization of fertilizer nutrients, since the growth of plants and their quality are mainly a function of the quantity of fertilizer. Table 6 shows the results of the fertilization tests on barley over the course of 21 days (carried out four times).

TABLE 6 FERTILIZATION TEST ON BARLEY CROP (21 DAYS AFTER PLANTING)

	Average stem length (cm)	Average root length (cm)
Control soil	17.83± 0.66	19.83± 0.03
Soil+Commercial fertilizer	22.16± 0.59	16.16± 0.10
Soil+ M5	24.50± 0.12	23.33±0.47

Fertilization tests were performed with M5 since it had the best physico-chemical results. The results of the test shows that M5 had optimal growth for the barley crop tested, which was better than the commercial fertilizer (Fig. 9).This mixture allowed an improvement in the lengths of the barley's stems and roots.

Fig 9. Fertilization test on barley crop (a) 8 days (b) 21 days.

International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
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IV.CONCLUSION

This study proposes a way of valuing abundant waste and pollutants from part of the food industry in Morocco. It shows the possibility of producing a good quality agricultural fertilizer from a ternary mixture of industrial waste, using response surface approach to optimize formula.

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