Biological synthesis of pharmacologically active polysaccharide

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ABSTRACT

Levan is produced by a species of *Bacillus* in a tightly controlled fermentation of sugar .Aims of this work to valorize agro waste in producing levan (the fructooligosaccharide) and optimize fermentation conditions for microbial production of levan polymer using respons surface methodology. The variables studied were pH, temperature, inoculum size, incubation period and light using sugar cane molasses as substrate and under the optimal environmental condition determined in this experiment, we conducted another experiment to study the effect of another two variables tween 80 surfactant concentration and the dry weight of bagasse using sugar cane bagasse as substrate . Also to purify levan from Bacillus sp culture media, characterization the levan polymer by H^1 NMR spectroscopy and physicochemical properties and to estimate it as glucose using colorimetric phenol sulphuric acid method.

Keywords: Levan polymer, *Bacillus Subtilis*, Biotransformation, Agro waste, Identification, Estimation, Purification, Drying, Storage.

INTRODUCTION

Fructans are built up of fructose residues, normally with a sucrose unit (i.e. a glucose-fructose disaccharide) at what would otherwise be the reducing terminus. The linkage position of the fructose residues determines the type of the fructan. Linkage normally occurs at one of the two primary hydroxyls (OH-1 or OH-6), and there are two basic types of simple fructan **(Van den and Wim, 2013)** , levans are a group of fructans; polymers of fructose forming a non-structural carbohydrate, which in the case of levans can themselves link together to form super-molecules comprising even hundreds of thousands. The two main types of fructans are the levans with mostly ß (2→ 6) linkages and the inulins with ß (2→1) linkages **(Han, 1990).**

Levan was first identified over 100 years ago. Properties distinguishing levan from other polysaccharides have long interested researchers. Its value in medical applications has been demonstrated repeatedly. But levan has only been available in small quantities and its potential has been unrealized because of the high cost. A method has been developed for large scale production, making levan economically viable (www.polysaccharides.us). Unlike polysaccharides used as emulsifiers and thickeners. Prebiotic effect of levan and its applications Levan has numerous demonstrated uses in foods **(JOGDAND, 2014)** levan is non-toxic, non-mutagenic, odorless, tasteless, and a soluble dietary fiber. Levan has been shown to exert excellent cell proliferating, skin moisturizing and skin

irritation-alleviating effects as a blending component in cosmetics. Levan derivatives, such as sulphated, phosphate or acetylated levans, are asserted to be anti-AIDS agents. In addition, levan is used as a coating material in a drug delivery formulation. Levan has a number of industrial applications, such as a surfactant for household use and a glycol/levan aqueous two-phase system for the partitioning of proteins **(Gupta et al., 2011).**

Levan is a diversely distributed component, particularly in plants, yeasts, fungi and bacteria **(Jang et al., 2002).** Bacterial species, such as Zymomonas mobilis, Bacillus subtilis, Bacillus polymyxa and Acetobacter xylinum produce extracellular levan **(Dina et al., 2007)**. Levans produced in grasses (Dactylis glomerata, Poa secunda and Agropyron cristatum) are present as storage carbohydrates in the stem and leaf sheaths and are degraded in the latter stages of the growing season to provide plants with carbohydrates for grain filling **(Ploolock and Cairns 1991)**. Substrates are sucrose or sucrose containing substances as some agro waste of sugar cane. Sugarcane processing produces cane sugar (sucrose) from sugarcane. Other products of the processing include bagasse, molasses, and filter cake **(Narendra and Yang, 2005).** The main objectives of our results to valorize agro waste in producing levan (the fructooligosaccharide) and optimize fermentation conditions for microbial production of levan polymer using respons surface methodology.

MATERIALS AND METHODS

Microorganism

Isolation and identification of *Bacillus subtlus* were done by collect bagasse samples from different regions of El Sadat city, Egypt; .1gm of bagasse sample was taken in 25ml conical flask containing 10 ml of sterilized water and contents were mixed well in vortex to get homogeneous suspension. The suspension is serially diluted 10^{-6} times and using streak plate technique the diluted samples are transferred to petri-dishes containing sterile skim milk agar medium. After inoculation the plates were incubated at 37ºC for 24 hours. After incubation bacterial colonies appearing over skim milk agar medium were identified based on colony characteristics and their identities were confirmed through Gram staining methods and by a series of biochemical tests as prescribed by Bergley manual and API System.

Substrates

Molasses

Black strap cane molasses obtained from sugar refinery factory at El- Hawamdia purified by addition of water in a ratio of 1:1, acidified to pH 4.0, heated in a water bath at 100ºC for 1 hour and kept overnight to precipitate the undesirable metal salts.

Bagasse

Sugarcane samples of different varieties were collected randomly from markets and the sugarcane juice was extracted for 30 min using a conventional juicer machine. The bagasses samples were collected and remaining fibres were subjected to further

extraction till four cycles of extractions were completed

Drying of bagasse

Since bagasse is a loose material, almost in shredded form; it is expected that it can be dried easily using microwave oven. A household oven has been used for this purpose. About 10g sample was taken in petty dish and was dried for seven minutes; the exposure time was segmented into different steps. At first step the bagasse was exposed for four minutes and was kept in a desiccator having silica gel for cooling. After taking the weight on an electronic balance, it was again exposed six times for 30 seconds each. Every time the weight was recorded after keeping and cooling it in desiccator. It was observed that the weight of sample becomes almost constant. So a total exposure time was taken as seven minutes.

Statistical media optimization

Statistical media optimization is a process whereby the initial screening of the factors possible to affect the weight of the yield is examined using Plackett-Burman experimental design to understand the significance of their effect on the Levan production and then optimization of significant factors using central composite rotatory design (CCRD).

Plackett-Burman design

The goal is to find experimental designs for investigating the dependence of the measured quantity on a number of independent variables (factors), in such a way as to minimize the variance of the estimates of these dependencies using a limited number of experiments using molasses as substrate table (1). Interactions between the factors were considered negligible.

Table (1) represents the factors as well as the testing levels of each factor in the experimental design using molasses as substrate

Light	Inoculum size	PH	Time	Temperature	parameter
X5	X4	X3	X2	X1	symbol
light	1.5 ml	5	48 hr	35	Low level
No light	2 ml	7	72 hr	42	High level

In the present work, five assigned variables (temperature, incubation time, pH and volume of inoculum) were screened in 12 experimental trials. All experiments were carried out in triplicate and the average of levan volumetric yield was taken as the response (dependant variable).

Response surface methodology

A central composite rotatory design (CCRD) for the effective 4 independent factors was used to obtain the combination of values that optimize the response within the region of three dimensional observation spaces, which allows one to design a minimal number of experiments. The experiments were designed using the software, SAS JMP 8 NULL. The independent variables selected for the optimization were (temperature, pH, and inoculum size & incubation period) as shown in table (2) .

Table (2) illustrates the factors investigated as well as the testing levels of each factor

Factor	Symbol	Low level	Med level	Max level
Temperature	X1	32	36	40
Time	X2	48	66	84
PH	X3	6	7.5	9
Inoculum size	X4	1	2	3

The behavior of the system was explained by the following second order polynomial equation :

$Y=\beta 0 + i = \Sigma \beta iXi + \Sigma \beta iiXiXj + \Sigma i\Sigma j \beta ijXiXj$

Y is the predicted response; β0 a constant; βi the linear coefficient; βii the squared coefficient; βij the cross-product coefficient. Regression analysis was performed on the data obtained from the design experiments. SAS JMP 8 NULL program was used for analysis of obtained data.

Media used

Activation media

1% Dextrose, .5% peptone and .45% yeast extract for the growth. pH should be 7-7.2 and temperature 30-37 c and reactivated using the same media every 24 hour. Biotransformation media using molasses as C source: 2% Cane molasses, 1% Dextrose, .5% peptone, .45% yeast extract and .7% NaCl.

Biotransformation media using bagasse as C source

The experiment intended to examine the following weights 1 gm, 2 gm, 3 gm, 4 gm, 5gm and 7 gm but virtually only 1 gm, 2 gm and 3 gm are applicable as other weights absorb the whole water of media leaving either meager amount of liquid or no liquid so the experiment conducted using the three suitable weights. The use of the non-ionic surfactant Tween 80 during pretreatment of sugarcane bagasse can enhance delignification and improve enzyme hydrolysis or lower the enzyme loading to achieve high sugar yields.Surfactants have been used in biotechnological processes to improve the production of number of biomolecules produced by fermentation Proposed mechanisms for increased production of biomolecules in presence of surfactants are increased cell membrane permeability, change in lipid metabolism and stimulation of the release of bio molecules. Cane bagasse, Tween 80, 1% Dextrose, .5% Peptone, .45% yeast extract and .7% NaCl.

We examined the suitable weights of bagasse that allow significant amount of liquor to be available and tween 80 concentration. Using central composite design (table (3)). The experiments were conducted under the optimal conditions assigned in the previous work (which had been performed using molasses as substrate); these optimal conditions were (Temperature 36 o c, pH 7.5, incubation time 66 hours and inoculum volume 2ml).

Table (3) central composit design with real values for Levan production using bagasse

Weight gm	3	2	3	1	2	3	1	2	1	2
Tween conc %	1	.75	.75	.5	1	.5	.75	.5	1	.75

Sterilization of culture media

Due to sensitivity of carbohydrates to heat especially in the presence of protein (yeast extract and peptone) since either maillard reaction, carmellization reaction or both can be developed so must set the autoclave at relatively low temperature or time. Either 115 o c for 20 minutes or 121 for 5 minutes are possible .It is more suitable as heating under pressure is an effective method for delignification.

Inoculum preparation

Inoculum for screening of Levan production capability using *Bacillus subtilis* and Cane molasses as substrate:

Scrape the frozen surface of the culture with a sterile inoculating loop, immediately streak the bacteria onto the surface of an LB agar plate containing the appropriate antibiotics, Incubate the plate overnight at 37 ^{O} C. Test tubes containing activation medium at pH 7 were sterilized at 121o c for 20 minutes, under complete sterile condition and by loop we inoculated some test tubes which were left for two days and then another sterile tubes were inoculated for reactivation of the maintained cultures. The assigned volume stated by the design was used to inoculate biotransformation media.

Filtration of media containing sugar cane bagasse as substrate

The culture media were poured into conical flasks through funnels covered with ordinary filter papers.

Levan precipitation

Samples collected were centrifuged at 4°C (10000 rpm, 20 min). The pellets were used as source of cell dry weight, decanted and the supernatant was then decanted and next 4 volumes of chilled 95% ethanol was added to it in relation to 1 sample volume, mixed and left for the night at 4°C. The liquid from the precipitated sediment was decanted and centrifuged with the aim to recover the low-density fraction suspended in it (10000 rpm, 20 min), the precipitates were mixed together and solved in deionized water and then heated in a boiling water bath for 10 min in order to deactivate exo-enzymes, cooled down and the levan was precipitated again. The obtained light-cream precipitate was dried at 60 degree C and stored at the desiccator **(Szwengie et al., 2004).**

Purification of Levan

Levan was dissolved in distilled water and repreciptated by the same procedures described above this process repeated several times until obtaining amorphous or microcrystalline, white powder, soluble in cold water, very soluble in hot water, insoluble in 75% alcohol.

Colorimetric analysis

Phenol Sulphuric Acid Method

Table (4) procedures of phenol sulphuric acid method (DuBois et al., 1956).

Tube No.	Blank	1	2	3	4	5	6	7
Conc. of Glucose (µg)	0.0	20	40	60	80	100	Test sample 1	Test Sample 2
Volume of Glucose stock taken (µl)	0.0	20	40	60	80	100		
Volume of distilled water added (µl)	100	80	60	40	20	0	100 µl	100 µl
Volume of 5 % Phenol solution added (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Volume of conc. Sulphuric acid added (ml)	1	1	1	1	1	1	1	1
Keep at room temperature for 10 minutes, Mix well and place in a water bath at 25 – 30^{0}C for 20 minutes								
Absorbance at 490 nm								

RESULTS AND DISCUSSION

Evaluation of factors affecting levan production (Plackett-Burman design) using molasses as substrate-:

Data in table (5) represents the actual design values and the results of

Different trials (response) in gm/L. The data given in table (5) showed a wide Levan yield variation (1.1 to 5.5 gm/L), thereby reflecting the importance of the environmental factors for attaining higher productivity.

Table (5) represents the actual design values and the results of different trials (response) in gm/L using (Plackett-Burman design)

temp	time	ph	inoculum	light	Y	Predicted Y
42	48	7	3	1	3.1	3.222222
35	48	5	3	0	2.4	2.622222
42	72	5	1.5	0	2.2	2.266667
35	72	7	3	0	5.3	5.511111
42	48	5	1.5	1	1.2	1.133333
42	72	7	1.5	0	3.7	3.611111
35	48	7	1.5	0	3.6	3.222222
35	72	5	1.5	1	3.5	3.833333
42	48	5	3	0	1.5	1.466667
35	72	5	3	1	5.1	4.577778
35	48	7	1.5	1	3.5	3.633333
42	72	7	3	1	4.46	4.766667

The main effects of the examined factors on levan production were calculated and presented in Table (6). On analyzing the regression coefficients for the five variables, the following four variables; temperature, time,

PH and incubation period, had effects on levan production, whereas light did not.

Table (6): The main effects of the examined factors on levan production

Prob>|t|	t Ratio	t Ratio	Std Error	Estimate		Term
0.0003*		7.48	0.09987	0.7466667		time(48,72)
0.0006*		6.48	0.09987	0.6466667		ph(5,7)
0.0009*		-6.04	0.09987	-0.603333		temp(35,42)
0.0133*		3.47	0.09987	0.3466667		inoculum(1.5,3)
0.1216		1.80	0.09987	0.18		light(0,1)

Results of the central composite rotatory design (CCRD) using molasses as substrate:

Based on the Plackett-Burman design; temperature, the incubation period (hours), PH and inoculum size were selected for further optimization of levan production using RSM. To examine the combined effect of these independent variables on the levan production, a central composite factorial design (26 experiments) were performed. The CCRD matrix of the independent variables in the form of the actual values, along with responses of each experimental trial, is given in Table (7).

Table (7) The CCRD matrix of the independent variables in the form of the actual values, along with actual and predicted responses of each experimental trial:

X1	X2	X3	X4	Y	Predicted Y
36	66	7.5	2	5	4.997381
40	48	6	3	1.5	1.724311
32	84	9	1	2.3	2.29839
40	48	6	1	1.3	1.017598
36	66	7.5	2	4.7	4.997381
40	66	7.5	2	3.5	3.877726
40	84	9	3	2.5	2.49839
36	66	6	2	4.4	4.457354
40	48	9	3	1.2	1.050931
36	66	7.5	1	4.1	3.977726
32	48	6	1	1.5	1.724311
36	48	7.5	2	4	3.957354
32	48	9	1	1	1.100931
32	84	6	1	2.5	2.450931
32	84	6	3	3.5	3.707645
40	84	6	3	3	2.700931
40	84	6	1	2	2.115056
40	84	9	1	1.8.	2.783353
32	48	9	3	1.5	1.607645
36	84	7.5	2	5.1	5.044393
32	48	6	3	3.3	3.101863
36	66	9	2	4.2	4.044393
36	66	7.5	3	4.5	4.52402
32	66	7.5	2	4.8	4.32402
40	48	9	1	1.2	1.215056
32	84	9	3	2.6	2.684265

The table showed different results when temperature, incubation period, PH and inoculum volume had changed and the following table showed that these factors have high significant effect. The interaction between these four factors appeared significant in many cases as we can see in the following tables table (8).

Table (8) Sorted Parameter Estimates:

Prob>|t|	t Ratio	t Ratio	Std Error	Estimate	Term
<.0001*		7.03	0.077299	0.5435196	X2(48,84)
0.0008*		-4.71	0.190287	-0.896508	X1*X1
0.0029*		-3.92	0.190287	-0.746508	X3*X3
0.0029*		-3.92	0.190287	-0.746508	X4*X4
0.0054*		3.53	0.077299	0.2731471	X4(1,3)
0.0162*		-2.89	0.077299	-0.223147	X1(32,40)
0.0234*		-2.67	0.077299	-0.20648	X3(6,9)
0.0251*		-2.63	0.082703	-0.21771	X3*X4
0.0261*		-2.61	0.190287	-0.496508	X2*X2
0.0325*		2.48	0.082703	0.2052095	X1*X3
0.0701		-2.03	0.082703	-0.16771	X1*X4
0.1851		1.42	0.082703	0.1177095	X2*X3
0.2885		1.12	0.082703	0.0927095	X1*X2
0.7225		-0.37	0.082703	-0.03021	X2*X4

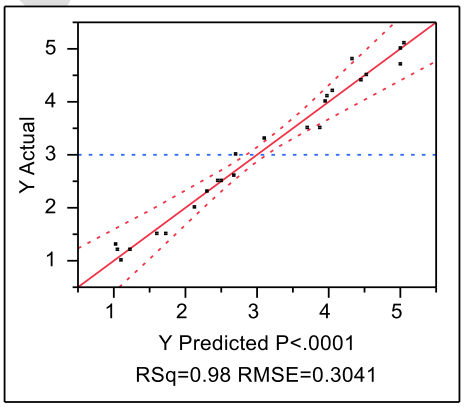
The corresponding second-order response model for CCRD after analysis of the regression is:

Y = 4.99 - .22 * [(x1-36)/4] + .543 * [(x2 - 66)/ 18 + .2 * [(x3 – 7.5) / 1.5] + .27(x4 – 2) + [(x1-36) /4] * {[(x2 -66) / 18]*.09 } + [(x1-36) /4] * {[(x3 – 7.5) / 1.5]*.2 } + [(x2 - 66) / 18] * {[(x3 – 7.5) / 1.5]*.1177 } + [(x1-36) /4] * [(x4 – 2) * - .16] + [(x2 - 66) / 18] * [(x4 – 2) * - .03] + [(x3 – 7.5) / 1.5] * [(x4 – 2) * - .2] + [(x1-36)/4] * {[(x1-36)/4] * - .8 }+ [(x2 - 66) / 18] * {[(x2 - 66) / 18] * -.4 } + (x4 – 2) * [(x4 – 2) * -.74]

Verification of model

The optimal conditions realized from the optimization experiment were verified experimentally and compared with the data calculated from the model figure (1) . The estimated levan yield was 4.8 gm/L, whereas the predicted value from the polynomial model was 4.99 gm/L, thereby confirming the high accuracy of the model. The RSq value for the levan production was .961443for Plackett-burman design. 979566 for CCRD This indicated a high degree of correlation between the experimental and predicted values and also indicated an increase in validity of experimental designs results.

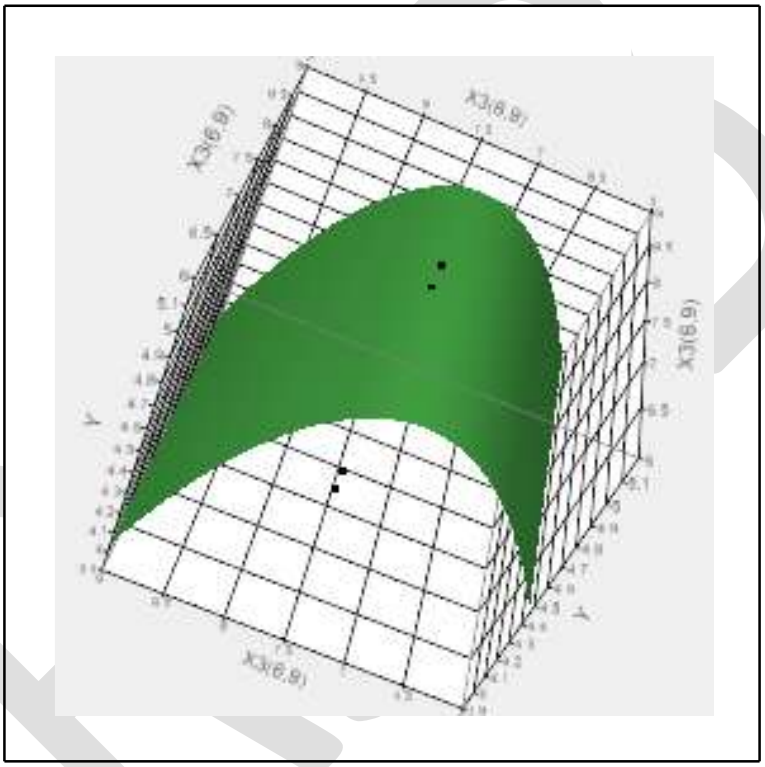
Fig (1) shows actual by Predicted Plot



Effect of the pH

Changes in the pH affect many cellular processes; such as regulation of the secondary metabolites biosynthesis and the slightly negative effect of PH is consistent with findings of **(Noorhisham et al., 1999)** that stated on the maximum growth of bacillus subtilis is at PH 6.5. The maximum concentration of levan obtained at pH 7.5 (figure (2)) no levan production occur at acidic pH as 4 and this attributed to inhibited cell growth although the sucrose hydrolyzing capacity is not inhibited **.**

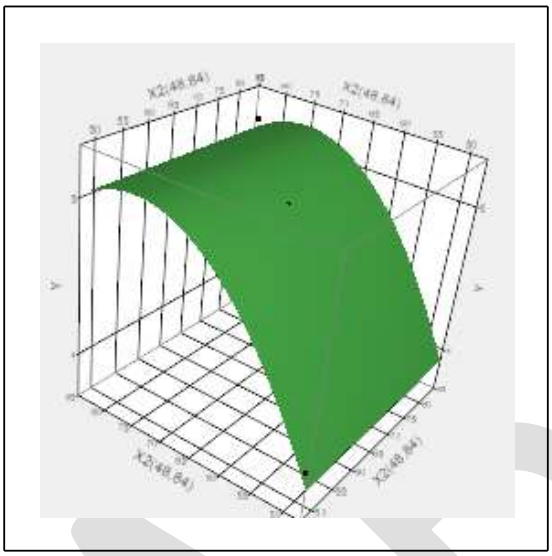
Fig (2) 3D surface plot for levan production -Relation between levan yield (gm/L) and pH.



Effect of the incubation period

Incubation period had a positive effect on the levan production as shown in figure (3), this may be attributed to that the bacteria was not completely active and the activation series were not enough that the inoculum, which might be unable to utilize the complex substrate, needed additional time.

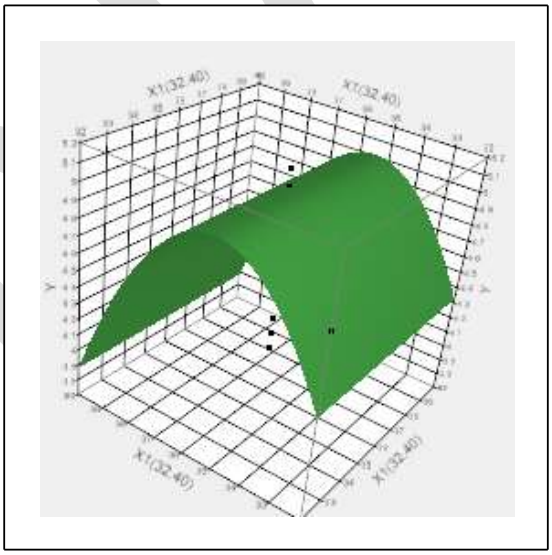
Fig (3) 3D surface plot for levan production -Relation between levan yield (gm/L) and incubation period



Effect of temperature

Maximum concentration of levan was obtained at 36^{O} C at PH 7.5 And this negative effect of temperature is consistent with findings of **(Sirajunnisa et al., 2013)**

Fig (4) 3D surface plot for levan production -Relation between levan yield (gm/L) and temperature.



Effect of the inoculum size

The effect is weak positive since it becomes negative after reaching the optimal volume (figure (5)) due to the depletion of nutrients by fast-growing of bacteria, and/or the formation of an inhibitory product such as an organic acid. Observations, however, that inoculum size could have an effect on the

duration of the lag phase have been reported **(Augustin Et al., 2000)**; since secondary metabolites (as levan) are produced within or at the end of the stationary phase **.**

Fig (5) 3D surface plot for levan production -Relation between levan yield (gm/L) and inoculum size

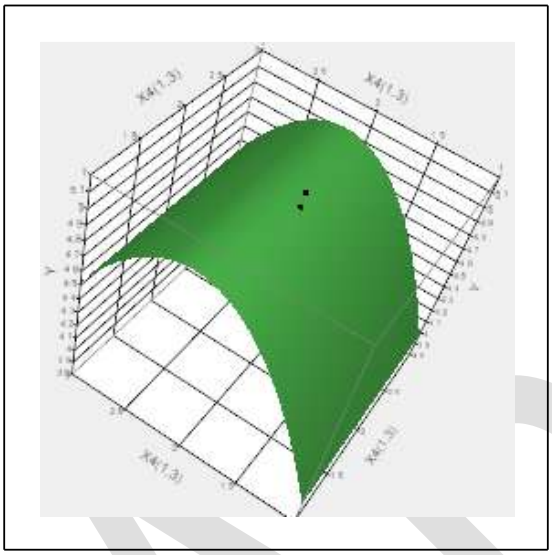


Table (9) Results of the central composite rotatory design (CCRD) using bagasses.

weight	tween conc	Y	Predicted Y
3	**1**	**2.1**	**2.120238**
2	**0.75**	**1.6**	**1.557143**
3	**0.75**	**2.3**	**2.242857**
1	**0.5**	**0.7**	**0.686905**
2	**1**	**1.4**	**1.409524**
3	**0.5**	**1.8**	**1.836905**
1	**0.75**	**1**	**1.042857**
2	**0.5**	**1.2**	**1.17619**
1	**1**	**0.9**	**0.870238**
2	**0.75**	**1.5**	**1.557143**

Based on the central composite rotatory design (CCRD) using bagasse: weight of bagasse and tween concentration have significant impact on the product , while the interaction between the two factors are not significant, and this can be observed in parameter estimates table (10) and figure (6)

Table (10) parameter estimates of (CCRD) using bagasse

Prob>|t|	t Ratio	Std Error	Estimate	Term
<.0001*	44.54	0.034962	1.5571429	Intercept
<.0001*	25.12	0.023884	0.6	weight(1,3)
0.0081*	4.88	0.023884	0.1166667	tween conc(0.5,1)
0.4409	0.85	0.029252	0.025	weight*tween conc
0.0888	2.24	0.038299	0.0857143	weight*weight
0.0023*	-6.90	0.038299	-0.264286	tween conc*tween conc

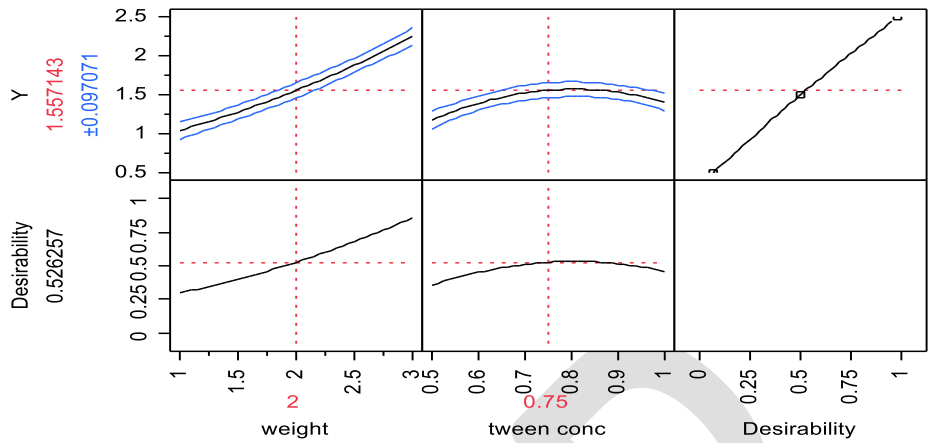


Figure (6) Prediction Profiler

Estimation of levan as glucose using Phenol Sulphuric Acid Method:

Table (11) results of spectrometric analysis of different concentrations of standard glucose

conc	abs
20	1.998
40	3.995
60	5.994
80	7.997
100	9.996

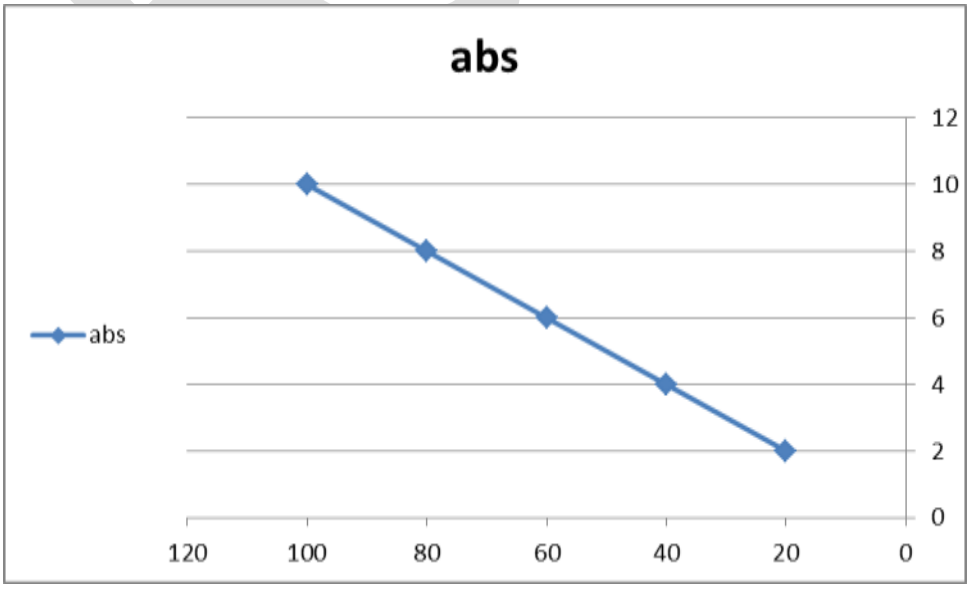


Figure (7) Standard Curve for Total Carbohydrates by Phenol Sulphuric Acid Method.

Interpretation

The Phenol Sulphuric Acid method is carried out by preparing a set of solutions with known glucose concentrations and mixing them with the Phenol- Sulphuric acid reagent. A standard curve can be made and the concentrations of unknown sugar samples can be derived from the standard curve.

REFERENCES

Aly Savadogo, Cheik W. Savadogo, Nicolas Barro, Aboubakar S. Ouattara and Alfred S. Traore. 2004. Identification of exopolysaccharides producing lactic acid bacteria from Burkino Faso fermented milk samples. African Journal of Biotechnology, 3. 189-194.

Augustin, J-C., Brouillaud-Delattre, A., Rosso, L. and Carlier, V. 2000. Significance of Inoculum size in the lag time of Listeria monocytogenes. Applied and Environmental Microbiology, 66. 1706-1710.

Dina, R., A.K. Pal, Z.P. Bhathena, N.P. Sahu, A. Jha and S.C. Mukherjee. 2007. Dietary Microbial Levan Enhances Cellular Non-specific Immunity and Survival of Common Carp (Cyprinus carpio) Juveniles. Fish & Shellfish Immunology, 22. 477-486

DuBois, M., Gilles, K., Hamilton, J., Rebers, P., & Smith, F. 1956. Colorimetric methodfor determination of sugars and related substances. Analytical Chemistry, 28(3). 350–356.

Gupta , S. K. pronobda s, s. K. Singh , M. s. AKhtar , d. K. Meena and s. c. Mandal. Microbial levan. An ideal prebiotic and immunonutrient in aquaculture Aquafeed Product Manager, Guabi Animal Nutrition Member of the National Organizing Committee of World Aquaculture 2011.

Han, Y.W. 1990. Microbial levan. Advances in Applied Microbiology 35:171-194.

Jang, K. H., S. A. Kang, Y. H. Cho, Y. Y. Kim, Y. J. Lee, K. H. Hong,K. H. Seong, S. H. Kim, C. H. Kim, S. K. Rhee, S. D. Ha and R.W. Choue. 2003. Prebiotic properties of levan in rats. Journal of Microbiology and Biotechnology, 13:348-353.

JOGDAND, S.N.2014. Biosurfactants and Bioemulsifiers. Biopolymers. Biotechnology support service (BSS). India, page 46.

Narendra, R. and Y. Yang. 2005. Biofibers from agricultural byproducts for industrial applications. Trends in Biotechnology, 23. 22-27.

Noorhisham Tan Kofli, and Mohd. Sahaid Kalil. 1999. Effect of pH and agitation on growth of bacillus subtilis. Sains Malaysiana, 28 .49-57.

Ploolock, C. J. and A. J. Cairns. 1991. Fructan metabolism in grasses and cereals. Annual Review of Plant Physiology and Plant Mollecular Biology, 42. 77-101.

Sirajunnisa Abdul Razack, Vijayagopal Velayutham and Viruthagiri Thangavelu. 2013. Influence Of Various Parameters On Exopolysaccharide Production From Bacillus. International Journal ofChemTechResearch,5.2221-2228

. Szwengiel A. , Czarnecka M. , Roszyk H. , Czarnecki Z. 2004. Levan Production By Bacillus Subtilis DSM 347 Strain, EJPAU 7(2).

Van den Ende, Wim. 2013. Multifunctional fructans and raffinose family oligosaccharides". Frontiers in Plant Science4.- doi:10.3389/fpls.2013.00247

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