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Effect of Thermodynamic Parameters Variation on Cell Growth of Azotobacter Specie for Bio-fertilizer Applications

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Abstract
The effect of changes in thermodynamic parameters on cell growth of Azotobacter specie was investigated. Isolation and confirmation of Azotobacter species was also carried out. This was done by identification of their external morphology, coupled with biochemical confirmatory tests. Central Composite Design of the Response Surface Methodology was employed in the design to investigate the influence of covariates variables (Temperature and Stir rate) on the criterion variable (cell growth). Stuart Orbital Shaker incubator was used in experimental culturing of Azotobacter microbe in line with the designed outlay that required 13 runs. In each case, temperature was altered from 29^0C to 37^0C and stir rate altered from 138rpm to 222rpm sequentially within 48hours while the cell growth determined through the viable cell count technique. Impacts of other thermodynamic parameters like pressure, enthalpy and entropy, on cell growth were equally detected. Azotobacter was revealed to have quadratic increase in growth rate from 2E+07Cfu/ml to 4E+07Cfu/ml in response to linear temperature increase from 29^0C to 37^0C, linear growth increment from 2E+07Cfu/ml to 3E+07Cfu/ml in response to linear stir rate increase from 138rpm to 222rpm and exponential growth increment from 2E+07Cfu/ml to 6E+07Cfu/ml in response to the combined factors. An improved design of culturing equipment that incorporate and make provision for the direct control of the enumerated thermodynamic parameters was then recommended.

Keywords: Isolation, Response surface methodology, Cell growth, Azotobacter, Temperature, Stir rate

1. Introduction

The entire functions of bio-fertilizers based on certain deductions are captured on the roles of the microbial contents (Elavarasi, Yuvaraj and Gayathri, 2020; Ramasamy, Geetha and Yuvaraj, 2020; Asadu et al., 2020, and Mitter et al., 2021). Once the useful microbe is grown, emphatically, a bio-fertilizer type had been formulated (Fraile, Menéndez and Rivas, 2015; Sulewska et al., 2019; Nosheen, Ajmal and Song, 2021; Koskey et al., 2021). All other components of bio-fertilizers were there either to; nurture, provide favourable environment or prolong the shelf life of the microbes (Hassan & Bano, 2015; Adnan et al., 2014; Kaljeet, Keyeo and Amir, 2011). Sometimes, some of the carrier materials were there to put it in acceptable appearance or to enhance easier application of the product. As microbial content of the bio-fertilizers is of utmost important, its indispensability in bio-fertilizers production process cannot be over emphasized. Effort is usually geared towards devising a means of its faster proliferation that is regarded as microbial growth. Microorganisms are similar to more complex organisms in that they need variety of materials from their environment to function and accomplish two primary goals, viz; supply enough energy to manage their processes and extract building blocks to repair themselves or procreate (Lacoma, 2018; Iweriolor& Okonkwo, 2014).

For rapid growth of the microbes to be achieved, there is need to fundamentally determine the parameters that influence the cell growth. Airtek Environmental Corp (2017), Lacoma (2018), Michigan State University (2020) and Kaiser (2021) and Okonkwo and Iweriolor (2014) identified warmth, moisture, pH levels and oxygen level and bio-films as the five big physical and chemical factors that affect the microbial growth. From their explanations, temperature of an area could be a massive contributor to microbial growth. Equally, Onokwai et al (2019) and Okonkwo et al. (2019) noted the influence of temperature towards growth, Also, bacteria thrive in warmth, growing mostly in areas close to temperature of human's body. Consequently, cooler locations tend to slow growth of microbes, as seen when food is refrigerated to keep it safe to eat longer. Likewise, they went further by stating that

the pH level of an environment could either help or hurt the growth of microbes. In addition, that microbes tend to prefer pH levels that are neutral and often harmed when more basic or acidic elements are present in a location. Moreover, they stated that oxygen-enriched locations and areas with vital nutrients will cultivate more microbial growth than locations with reduced oxygen levels. Based on that, controlling oxygen levels in an area could be difficult, but keeping areas clear of food and other sources of nutrients will starve out bacteria.

Observations made from reviewed research findings, indicated that the attention of the previous researchers are mostly focused on the manipulation of media (substrate) composition and in some cases, alongside with alterations in temperature or pH levels to affect cell growth rate (Zeng and Yang, 2020; Onokwai et al 2019; Okonkwo et al, 2019; Lele and Watve, 2014). However, Roden and Jin (2011), Ouldridge (2018) and Ahmad et al. (2020) stressed that thermodynamic parameters had influence on cell growth. Cells required energy for their proliferations which could be determined or computed with the help of various thermodynamic parameters like temperature, stir rate, pressure, enthalpy and entropy (Dragicevic and Sredojevic, 2011; Murphy, 2013; Popovic, 2019). Changes in temperature for instance is of optimum importance that microbes are classified as psychrophiles, mesophiles and thermophiles based on the temperature range they could thrive (Tankeshwar, 2019). Stir rate induces mobility, enabled percolation of oxygen and circulation of nutrients among others. In contrast to the previous empirical studies reviewed, the present study sought to determine the impacts of different thermodynamic parameters on cell growth of Azotobacter specie for bio-fertilizer applications.

2.0 Material and methods

1kg of clay soil mixed with compost manure from cultivated land was collected from a farm land in Awka, Anambra state of Nigeria and taken to the laboratory for isolation purposes.

2.1 Isolation of Azotobacter Specie and Confirmatory tests run

One gram of the soil sample was taken to the laboratory where it was suspended in 100ml of sterilized water in 500ml conical flask which served as stock solution. Nine (10ml capacity) test tubes that were filled with sterilized water up to 9ml each were used in the serial dilution. This was done by transferring 1ml of stock solution to the first test tube (10^{-1} dilution) with the help of a micro pipette. The serial dilution was iterated by transferring 1ml of each diluted test tube to the next until 10^{-9} dilution was achieved. In line with Mukhtar et al. (2018), a selective Burk agar medium termed; M1: (g/l) glucose 10.0; dipotassium hydrogen phosphate, 0.64; potassium dihydrogen phosphate, 0.16, NaCl, 0.2; $MgS0_4.7H_20$, 0.2; $CaSO_4$. $2H_20$, 0.05; NaM_o0_4. $2H_20$, 0.01; $FeSO_4$, 0.003, pH 7.1, was prepared and autoclaved at 121^{o}C (15lbf/inch^2 pressure) for 15 minutes. Then 0.1ml of the dilution was spread on the solidified surface of Burk agar medium plates that was incubated at 37^{o}C for 7 days. Azotobacter species were identified by their external morphology, coupled with biochemical confirmatory tests including; gram staining, methyl red test, catalase test, hydrogen sulphide test, indole test, motility test, protease test and endospore test, using routine methods in the laboratory as shown on Table 1.

Table 1: Morphological and biochemical characteristics of the isolated specimen

Test Category	Observation	Inference
Non - Microscopic		
Direct appearance on the plate	Yellowish brown, flat, slimy.	Azotobacter sp. suspected
Microscopic study		
Gram staining result.	Ovoid shaped and pink coloured.	Azotobacter sp. suspected
Biochemical Characteristics		
Gram reaction	Gram negative	Azotobacter sp. confirmed
Methyl red test	Positive	
Catalase test	Positive	
Hydrogen Sulphide test	Positive	
Indole test	positive	
Motility test	Positive	
Protease test	Negative	
Endospore test	Negative	

2.1 Experimental design for culturing the microbe

Two-levels, two-factors Central Composite Design (CCD) of Response Surface Methodology (RSM) was employed using Design Expert software version 11to determine the number of experimental runs and experimental permutations that were required to be conducted. Factors are selected after considering the microbial growth energy determinant thermodynamic parameters along with the controllable provisions made on the available laboratory culturing equipment (Stuart Orbital Shaker) while paying serious cognizance to the gap in the literatures reviewed. The corollary was then the development of experimental planto assess the influence of temperature changes (T), and changes in stir rate (N) on the cell growth (G). The variable levels were chosen after considering the surviving temperature range of Azotobacter specie following its mesophilic nature and the possible culturing stir rate interval based on the reviewed empirical experiences along with the laboratory recommendations. Supply of the low and high levels of the independent factors enabled the Design Expert software to display the arrangement of experimental matrix that were used in conducting the experiments. The actual design outlay used in the experiments was shown on Table 2.

Table 2: Actual design outlay for the experiments

Std	Run	Factor 1 A: Temperature (deg C)	Factor 2 B: Stir Rate (rpm)	Response 1 Cell Growth (Cfu/ml)
1	5	30	150	
2	12	36	150	
3	4	30	210	
4	2	36	210	
5	13	29	180	
6	7	37	180	
7	8	33	138	
8	1	33	222	
9	6	33	180	
10	11	33	180	
11	9	33	180	
12	3	33	180	
13	10	33	180	

2.2 Culturing experimental procedure

A special medium described by Mukhtar et al. (2018) at M2: (g/l) Ammonium Sulphate, 0.2; dipotassium hydrogen phosphate, 0.8; M_gSO_4, 0.2; C_aSO_4, 0.1, M_o Solution, 1ml (0.1mg/ml); $FeSO_4$, 1ml (1.0mg/ml), Mannitol 20.0, pH 7.1 as dissolved in 1000ml of distilled water. The solution was autoclaved at 121^{o}C (15lbf/inch^2 pressure) for 15 minutes. When cooled, 100ml of the medium was transferred into 500ml flask, inoculated by 1ml of starter culture through the help of micro pipette at approximately laminar air flow chamber. The flask was corked with thick cotton wool stopper and cultured in a Stuart orbital shaker with incubator and its temperature and stir rate setting altered according to the designed outlay for 48hrs per experimental run. After which the cell growth was measured in colony forming unit per millilitre (Cfu/ml) through viable cell count technique.

2.3 Procedure of conducting viable cell counting technique

After 48 hours of each experiment, the growth of the microbial species in each experiment was counted through viable counting technique. This technique started with the serial dilution of the culture. The hallmark of the process was that seven 10ml capacity test tube was each filled with distilled water up to 9ml level. Then 1ml of the culture in each experiment was transferred with the help of micro pipette to the first test tube, then 1ml of the first test tube transferred to the second and the iteration continued until the last seventh test tube. Then the suspension was then mixed with molten agar and poured into plate, allowed to solidify. The plate was then incubated under controlled conditions within 24hours. The colonies developed were observed without the aid of microscope. The counting assumption was that each bacteria colony formed was as a result of individual cells that had undergone cell division. Each group of cells was expected to produce each colony, therefore counting was conducted by counting the

numbers of colonies that formed and multiplied with the dilution factor which was 10^7 in this case. The computed values were eventually recorded in colony forming unit per ml (Cfu/ml).

3.0 Results and Discussion

3.1 Influence of Changes in Temperature and Stir Rate on Cell Growth

Experimental permutations of alterations in temperature from 29^0C to 37^0C alongside with changes in stir rate from 138rpm to 222rpm in culturing processes according to the designed outlay, resulted to various cell growth values.In order to have a better understanding of the effects of independent variables on the response variable, the trendswere sequentially rearranged and represented in three-dimensional plot of cell growth versus stir rate and temperature as shown in figure 1.

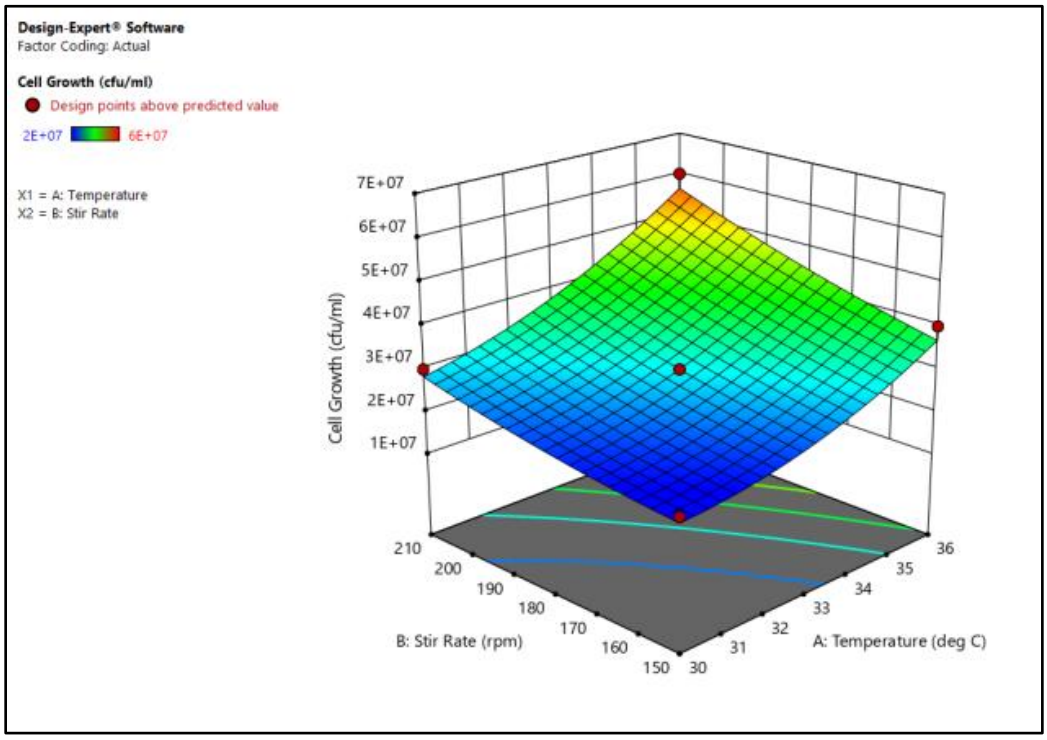


Figure1: Three-dimensional plot of cell growth versus stir rate and temperature

From figure 1, which is a 3D plot of Cell growth versus Stir rate and temperature changes, cell growth responses were seen to increase in reaction to positive adjustments in stir rate alongside with temperature changes. Quantitative explanations of the observed trends in figure 1. It was observed that when temperature was assumed to be constant, stir rate had a linear relationship with the cell growth such that linear increase in stir rate from 150rpm to 222rpm resulted to linear increase in cell growth from 2E+07Cfu/ml to 3E+07Cfu/ml.This occurred as result of the fact that stirring brought about agitation that enabled mixing of constituents, interaction of phases, fluidization, dissolution, atomization, emulsification, heat transfer, mass transfer and dispersion that favours absorption of oxygen (Couper et al., 2010) which resulted in cell growth.Similarly, when stir rate was assumed to be constant, temperature had a quadratic relationship with the cell growth such that linear increase in temperature from 29^0C to 37^0C resulted in quadratic increase in cell growth from 2E+07Cfu/ml to 4E+07Cfu/ml. This was based on the fact that increase in temperature increases molecular collision (Victoria State Government, 2020) thus reaction rate that favours cell growth.Those trends in the 3D plot of figure1 implied that changes in temperature had more impact towards determination of the cell growth rate than those of the stir rate. But cell grows in response to contributions of both factors. The curve of combination of linear increase in stir rate, from 150rpm to 222rpm and linear increase in temperature from 29^0C to 37^0C resulted in overall exponential increase in cell growth from 2E+07Cfu/ml to 6E+07Cfu/ml which was in line with the notion that bacteria grow exponentially (Shao et al., 2017; Allen and Waclaw, 2019).

3.2 Effects of variations in energy level on the considered thermodynamics parameters and cell growth

Following the cell growth responses of the experimental design outlay of figure 1, activation energy values, determined through standard procedures was plotted against the temperature and stir rate values as well as the cell growth values as shown in figure 2.

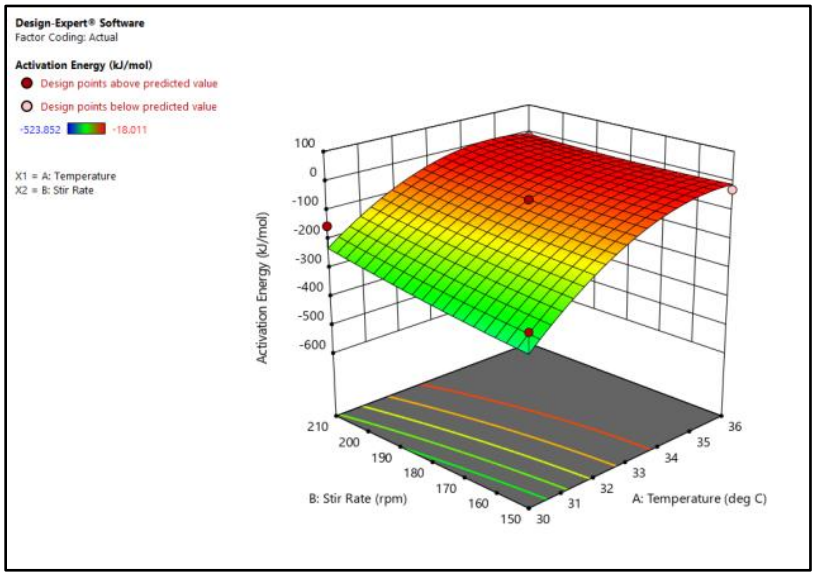


Figure 2: 3D Plot of activation energy versus stir rate and temperature

The trend in the graph of 3D plot of figure 2 was similar to those discussed in figure 1. When temperature was assumed to be constant and stir rate increased linearly from 150rpm to 222rpm, activation energy increased nearly linearly from -600kJ/mol to -240kJ/mol. Also, when stir rate was assumed to be constant, and temperature increased linearly from 29^0C to 37^0C, activation energy made a quadratic increase from -600kJ/mol to -18kJ/mol. But the overall graph of combination of changes in stir rate and temperature changes on the cell growth on the other hand showed a negative quadratic plot. The explanation of the phenomenon could be derived from the notion that temperature of a system is a measure of the kinetic energy of the molecules in the system (Puiu, 2020). Therefore, increase in temperature along with increase in agitation of molecules increases the activation energy that breaks the energy barrier which results in cell growth.

3.3 Generation of the statistical empirical model for predicting cell growth using Design expert v.11

Following the input of cell growth values as the response of the design outlay of the Design Expert v.11 software, ANOVA for quadratic model was generated and shown on Table 3.

Table 3: ANOVA for quadratic model *response 1:* Cell Growth

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.619E+15	5	3.239E+14	39.40	< 0.0001	significant
A-Temperature	1.068E+15	1	1.068E+15	129.91	< 0.0001	
B-Stir Rate	4.246E+14	1	4.246E+14	51.66	0.0002	
AB	2.500E+13	1	2.500E+13	3.04	0.1247	
A^2	9.783E+13	1	9.783E+13	11.90	0.0107	
B^2	1.087E+13	1	1.087E+13	1.32	0.2879	
Residual	5.754E+13	7	8.220E+12			
Lack of Fit	5.754E+13	3	1.918E+13			
Pure Error	0.0000	4	0.0000			
Cor Total	1.677E+15	12				

(Source: Design Expert v.11)

Table 3 showed the ANOVA for quadratic model of cell growth. Testing the significance of the model, it was observed as displayed by Design expert software that there is only a 0.01% chance that an F-value as large as 39.40 could occur due to noise. The model was therefore considered significant. Moreover, *P*-valuesless than 0.0500 indicated that model terms were significant. In this case A, B, A^2 were significant model terms. Values greater than 0.1000 indicate that the model terms are not significant. Coefficients of the expected model were equally tabulated as displayed by Design Expert v.11 software as could be seen on Table 4.

Table 4: Coefficients in terms of coded factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	3.000E+07	1	1.282E+06	2.697E+07	3.303E+07	
A-Temperature	1.155E+07	1	1.014E+06	9.156E+06	1.395E+07	1.0000
B-Stir Rate	7.286E+06	1	1.014E+06	4.889E+06	9.682E+06	1.0000
AB	2.500E+06	1	1.434E+06	-8.897E+05	5.890E+06	1.0000
A^2	3.750E+06	1	1.087E+06	1.180E+06	6.320E+06	1.02
B^2	1.250E+06	1	1.087E+06	-1.320E+06	3.820E+06	1.02

(Source: Design Expert v.11)

Considering Table 4, the coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs as indicated. The coefficients are adjusted around the average based on the factor settings. The variance inflation factor (VIF) as recorded by Design expert software indicated on the table, measured how much the variance around the coefficient estimate was inflated by lack of orthogonality in the design. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicated multi-colinearity, the higher the VIF the more severe the correlation of factors. The statistical model generated by Design expert software v.11 for the interaction above was presented in equation 1.

$$G(t) = 5.22950E + 08 - 2.86489E + 07 \times T - 1.17382E + 06 \times N + 4.16667E + 05 \times T^2 \quad (1)$$

It is important to note that temperature value in statistical model of equation (1) is in Celsius unit. The equation 1 could be used in making predictions about the response of a given levels of each factor. Being generated based on regression analysis, it was positioned to make predictions in line with the underlying experimental responses. Conversely to the models generated by Markov (2011) and Maitra and Dill (2015) that focused on substrate consumption rate, the generated model was based on manipulation thermodynamic parameters.

3.4 Prediction Comparisons

Comparison between the cell growth (G(t)) values predicted by the statistical model and actual value in figure 3 showed little deviations that were within the acceptable range. This may occur as a result of omission of various nearly useful equation parameters as a result of possession of insignificant P-values in Table 3.

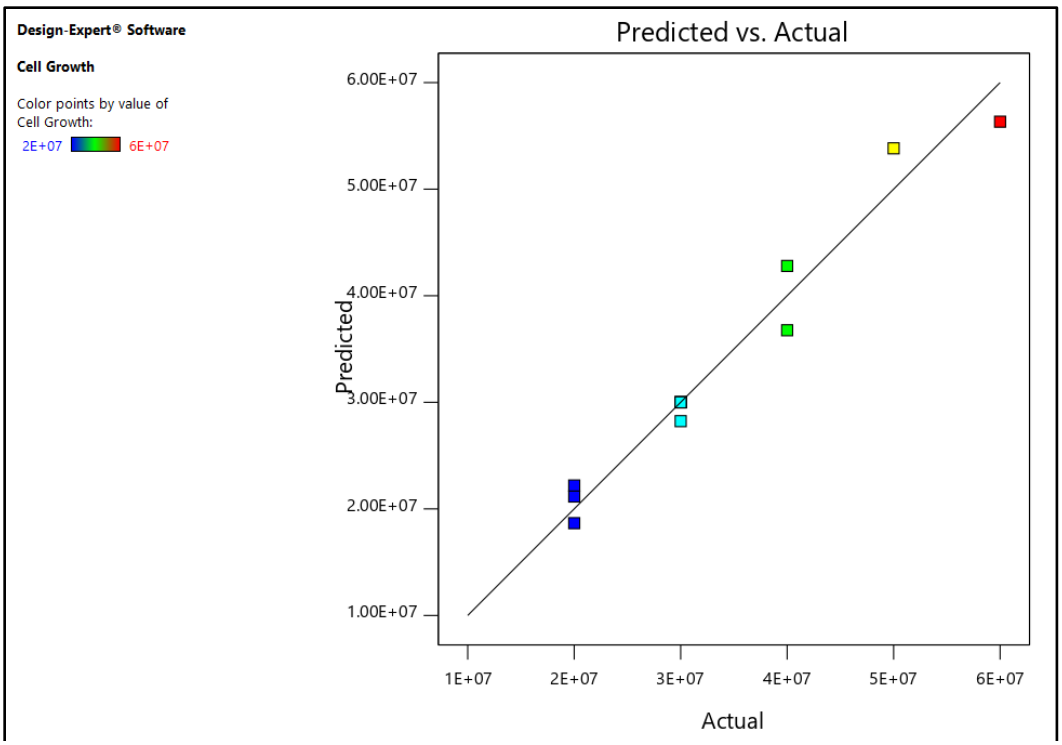


Figure 3: The graph of predicted versus actual cell growth.

3.5 Other thermodynamics parameters that could limit cell proliferation

In order to determine other thermodynamic parameters that could limit cell growth, The values of pressure, enthalpy, entropy and other derived parameters were plotted against their corresponding temperature values as shown in figures 3, 4, 5,6 and 7. This was based on the observations made from figure 1 that showed that temperature had a big impact on determination of microbial growth.

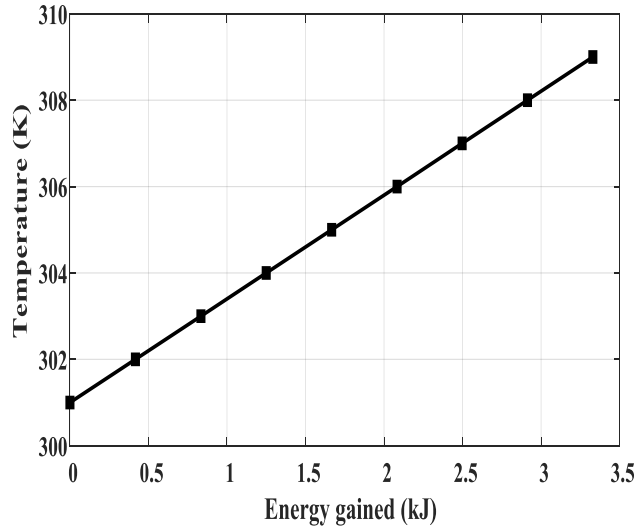


Figure 3: Energy gained versus temperature

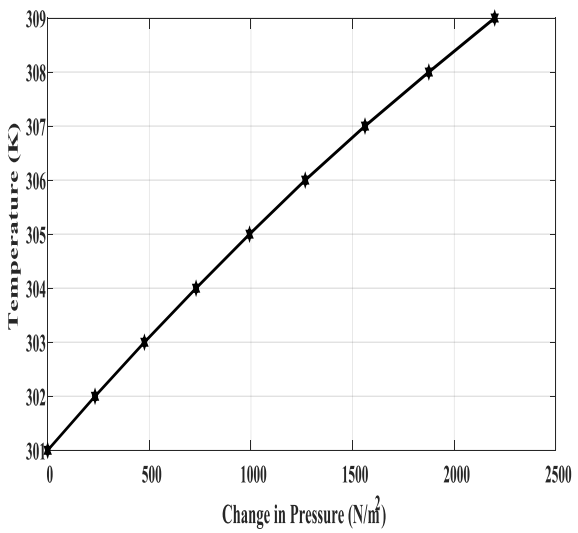


Figure 4: Pressure versus temperature

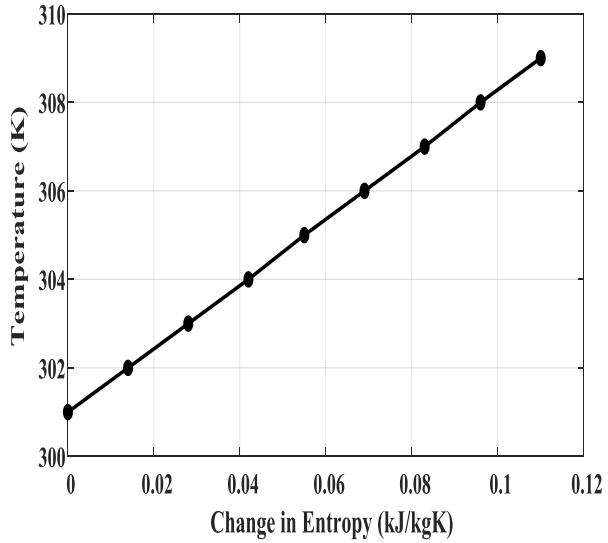


Figure 5: Entropy versus temperature

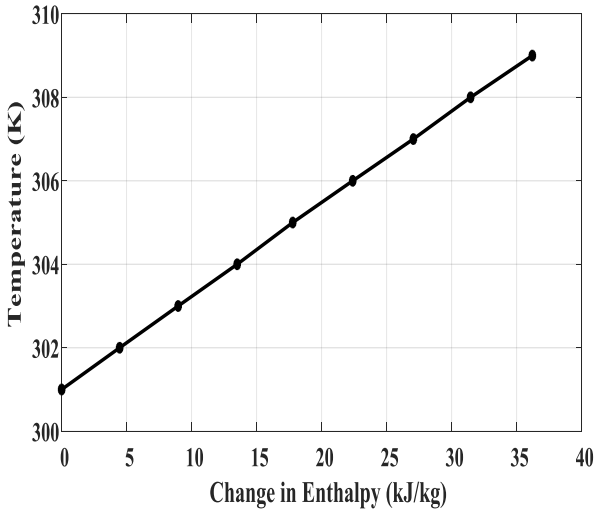


Figure 6: Enthalpy versus temperature

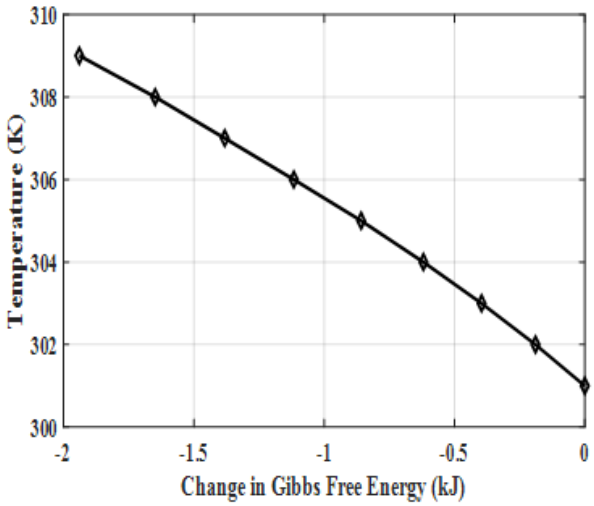


Figure 7: Gibbs free energy versus temperature

Having observed from figure 1, that temperature played greater role in determining the cell growth. It is then imperative to discuss other thermodynamic parameters that related to temperature so as to evaluate their relationship with the temperature on the condition that positive relationship would result to increase in cell growth whereas, negative relationship would result to decrease in cell growth. In line with the above, figure 3, that is a graph of heat gained versus temperature was plotted to determine the relationship between heat gained and temperature, it was observed that a linear positive relationship existed that showed that increase in heat gain within the mesophilic range would amount to increase in cell growth. Similarly, figure 4 which is a graph of change pressure versus temperature equally plotted also showed a positive linear relationship. Which proved that the experiment would have equally been conducted by varying the pressure although at non expense of the living conditions of the microbe. As the increase in pressure resulted to increase in temperature, it meant that increase in pressure will amount to increase in cell growth. Also figure 5 that is a plot of change in entropy versus temperature, showed that increase in the entropy favoured the increase in temperature and that would unquestionably have brought about the increase in cell growth. The change in entropy may be brought about by either increase in temperature or by increase in stir rate or both. Figure 6 similarly is the graph of change in enthalpy versus temperature. It could equally be seen that the increase in the enthalpy resulted to the corresponding increase in the temperature that would result to increase cell growth.

In the case of figure 7, which is the graph of change in the Gibbs free energy versus temperature, a different scenario emerged. It was observed that decrease in Gibbs free energy brought about proportionate increase in the temperature. That meant that decrease in Gibbs free energy would amount to increase in cell growth. In the present situation where both the change in enthalpy and change in entropy were positive, then change in Gibbs free energy would only be negative at higher temperature which determined the spontaneity of the reaction (Ck-12, 2013; Spontaneous Reaction: Definition and Examples, 2016;Ptaček et al., 2018; Soult, 2020) and increasing the growth rate.

3.5 Achievements of the study

- The study provided a way of fastening the bio-fertilizers production process by identifying the various thermodynamic parameters in which their appropriate manipulation could speed up the culturing period of the microbial inoculants.
- It presented energy interactions between the cell and its environment as the key growth determinant factor as against the previous studies that focused majorly on substrate consumption rate.
- It generated a statistical model that could predict the population of Azotobacter specie that could be cultured over a certain period in sufficient media concentration.

4.0 Conclusion

Azotobacter culturing experiments were conducted by following the two level, two factor central composite design of response methodology experimental design that required 13 runs. The temperature and stir rate that formed the independent variables were alternately manipulated according to the experimental design and the cell growth that formed the response, was then measured, various computations were made and different graphs plotted. The trends in the plotted graphs had shown that linear increase in culturing temperature from 29^0C to 37^0C resulted to quadratic increase in cell growth from 2E+07 Cfu/ml to 4E+07Cfu/ml. Similarly, that Azotobacter grow linearly from 2E+07Cfu/ml to 3E+07Cfu/ml in response to increase in stir rate between 150rpm and 222 rpm. That combination of the two factors within the designed outlay, brought about exponential increase in cell growth from 2E+07Cfu/ml to 6E+07Cfu/ml. The effect may be affected by the rate of activation energy utilization as observed from linear increase in stir rate from 150rpm to 222rpm that amounted to linear increase in activation energy from -600kJ/mol to 240kJ/mol. Likewise, linear increase in temperature from 29^0C to 37^0C brought about quadratic increase in activation energy from -600kJ/mol to -18kJ/mol. Overall plot of combination of the two factors against the activation energy however, showed a negative quadratic plot. In addition, that increase in; pressure, entropy and enthalpy increase the temperature and subsequently would equally increase the cell growth that would conversely be decreased by increase in Gibbs free energy.

5.0 Recommendation

Having identified that changes in; pressure, enthalpy, entropy and Gibbs free energy affect cell growth, there is need to design a culturing apparatus that would incorporate and make provisions for their alterations in order to directly observe their accurate responses on the cell growth.

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