

A Statistical Approach in Designing an Economically Viable Production Medium for *Acinetobacter junii* CN1 PHB

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ABSTRACT : With a view to design an economically viable production medium, the routine NLMS medium used in the production of the biopolymer , Polyhydroxybutyrate (PHB) with a bacterial isolate of a cashew nut industrial site, *Acinetobacter junii* CN1, was made cost effective by substituting with the cheap, renewable and locally available agro-wastes / resources. Besides, the media parameters / conditions / levels of nutrients / cultivation strategies were also statistically designed to optimize the PHB production. Subsequently, when an analysis was made on the production cost of PHB, with the medium designed in the present investigation, the cost is seemed to be relatively cheaper when compared with the experiments designed with the routine chemically defined medium. However, the cost worked out [₹ 198 /- (US \$ 2.93) per kg] does not include the manpower and other operational costs. On an average, even if US \$ 5 - 7 per kg is added as the operational cost , the production cost of PHB would be ranging between US \$ 8 and 10 only and thus proved to be economically viable.

KEYWORDS: Polyhydroxybutyrate, Statistical design, Cost effective medium. *Acinetobacter junii* CN1

I. INTRODUCTION

The production cost of commercially available microbial PHAs is approximately US \$ 16 per kg. This amount is comparatively 15 times higher than that of the conventional synthetic plastic, polypropylene [1]. Generally, the biopolymer producing microbes exploit glucose / sucrose as carbon source [2] [3] and the organic nitrogen compounds viz., amino acids and short peptides as nitrogen sources. These routine expensive media components tend to increase the production cost of the microbial polymer [4]. Thus it is evident that the major drawback in commercialization of biopolymer is mainly their high production cost, when compared with petrochemical plastics [5]. Hence, for the economical production of PHAs, cheaper renewable sources which could be used as the carbon feed stock and the bacterial strains able to produce large quantities of intracellular PHAs by exploiting the low - cost substrates are very much required. In this context, abundantly available wastes / resources generated through agricultural sector shall be exploited. Further, the efforts are also underway to substantially reduce the same by designing more efficient fermentation medium [6] [7] with improved conditions and cultivation strategies [8], down streaming processes [9][10] [11] and the use of inexpensive substrates [12] as well. Especially, exploitation of inexpensive carbon sources to produce PHAs has been reported with remarkable economical advantages [13].

In this context, the conventional approaches in which the fermentations are optimized empirically by using one-at-a-time variation of process parameters seem to be inefficient and time consuming. Moreover, it assumes that the various fermentation parameters do not interact and the process response is not a direct function of the single varied parameter. Further, it is evident that the observed behaviour of fermentation results from the interactive influences of the various variables [14] whereas the statistical optimization methods can correlate the interaction of variables in generating the process response. Factorial design of optimization experiments is especially suitable to account for the interactions, and its efficiency. A combination of factors (independent process variables) that could generate a certain optimum response can be identified through factorial design and the use of response surface methodology [15] [16] as

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well. It provides a systematic and efficient research strategy for studying the interaction effects of various parameters. In addition to that the use of friendly software packages has made this technique increasingly popular for media optimization [14].

Thus with a view to design a cost effective media in a most economically feasible way, the Taguchi method has been adopted to optimize the media parameters and the levels of nutrients [17].

II. MATERIALS AND METHODS

Designing Cost Effective Medium

With a view to design a cost effective medium for the production of the biopolymer PHB with a bacterial isolate of a cashew nut industrial site, *Acinetobacter junii* CN1, [18] the basic design of the NLMS medium was suitably modified by substituting the nutrient sources with the cheapest substrates at different levels (Table 1 & 2) which were then subsequently optimized at various conditions and levels of physicochemical variables (Table 3).

Table 1: Levels of substituted nutrient sources adopted for PHB production at various conditions

Conditions	Sources	Substitutes	Level 1	Level 2	Level 3
A	Carbon	Sago Waste	1.00%	2.00%	3.00%
B	Nitrogen	Corn Steep Liquor	0.10%	0.20%	0.30%
C	Citric acid	Lemon Juice	0.10%	0.20%	0.30%
D	Trace elements	Tender Coconut	1.00%	2.00%	3.00%

Table 2: Levels of physicochemical parameters adopted for PHB production at various conditions

Conditions	Sources	Level 1	Level 2	Level 3
A	pH	7	8	9
B	Temp (°C)	25	35	45
C	Incubation (hrs)	24	48	96

Table 3: Conditions versus Levels of nutrient substitutes / Levels of physicochemical parameters adopted for PHB production

Conical Flask	Conditions				Conditions		
	A	B	C	D	A	B	C
	Levels of nutrient substitutes				Levels of physicochemical parameters		
	1	2	3	4	1	2	3
1	1	1	1	1	1	1	1
2	1	2	2	2	1	2	2
3	1	3	3	3	1	3	3
4	2	1	2	3	2	1	2
5	2	2	3	1	2	2	3
6	2	3	1	2	2	3	1
7	3	1	3	2	3	1	3
8	3	2	1	3	3	2	1
9	3	3	2	1	3	3	2

In the present investigation, Taguchi method [17] was adopted not only to determine the optimal conditions / levels of nutrients and their substitutes in different combinations (Table 1 & 2) but also the various conditions / levels of physicochemical variables (Table 3). Further, the data pertaining to the process parameters were statistically treated with ANOVA to find out their level of significance in terms of their performance characteristics, which in turn were measured as signal to noise (S/N) ratio and grey relational analysis.

III. RESULTS AND DISCUSSION

In order to maximize the effectiveness of conversion of constituent biowastes / bioresources, the potential PHB producer, *Acinetobacter junii* CN1 strain [18] an isolate of cashew nut industrial site has been exploited. Further, as an attempt to minimize the production cost of PHB, the basic design of the medium has been tailor made in a cost effective manner by adopting Taguchi and Grey analysis methods as well [17]. Accordingly, the optimal conditions / level of nutrients / substitutes were determined (Table 4 - 6 ; Figure 1- 3) for the PHB production.

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Table 4 : S/N ratio of biomass and biopolymer for nutrient constituents

S/N Ratio of Biopolymer	Normalize	Coefficient	S/N Ratio of Biomass	Normalize	Coefficient	Grey Grade
5.145	0.225	0.535	-1.660	0.000	0.471	0.503
0.964	0.478	0.630	6.989	0.651	0.718	0.674
5.336	0.214	0.531	3.076	0.357	0.580	0.556
8.874	0.000	0.471	5.400	0.531	0.655	0.563
-1.663	0.636	0.710	6.572	0.620	0.701	0.705
-7.687	1.000	1.000	11.623	1.000	1.000	1.000
2.036	0.413	0.603	7.687	0.704	0.750	0.676
-3.733	0.761	0.789	8.369	0.755	0.784	0.786
2.545	0.382	0.590	5.778	0.360	0.699	0.630

Table 5 : Grading by Grey Analysis with Reference to S/N Ratio Nutrient Constituents

S. No.	C	N	CA	TE	Bio-polymer g/L	S/N Ratio of Bio-polymer	Biomass g/L	S/N Ratio of Biomass
1.	1	0.1	0.1	0.1	0.553	5.145	0.826	-1.660
2.	1	0.2	0.2	0.2	0.895	0.964	2.236	6.989
3.	1	0.3	0.3	0.3	0.541	5.336	1.425	3.076
4.	2	0.1	0.2	0.3	0.36	8.874	1.862	5.400
5.	2	0.2	0.3	0.1	1.211	-1.663	2.131	6.572
6.	2	0.3	0.1	0.2	2.423	-7.687	3.812	11.623
7.	3	0.1	0.3	0.2	0.791	2.036	2.423	7.687
8.	3	0.2	0.1	0.3	1.537	-3.733	2.621	8.369
9.	3	0.3	0.2	0.1	0.746	2.545	1.945	5.778

:Carbon;Nitrogen; CA: Citric Acid and TE: Trace Element)

Table 6 : Mean response for the nutrient constituents

Parameters	Table for Mean		
	1-MEAN	2-MEAN	3-MEAN
C	0.578	0.756	0.697
N	0.581	0.722	0.728
CA	0.763	0.622	0.646
TE	0.613	0.784	0.635
TOTAL	2.5339	2.8840	2.7067
MEAN GREY	0.677		

In the present investigation, the nutrients were substituted (glucose- sago; ammonium nitrate - corn liquor; citric acid - lemon juice; Trace elements - tender coconut) with the cheapest agro - industrial wastes / resources. Further, the analysis of data including interactions do also follow the same steps with the objectives to (i) determine the optimum condition (ii) identify the individual influence of each factor and (iii) to estimate the performance at optimum condition. However, to determine the optimum condition, the main effect and interaction effects have also been calculated.

It is worth mentioning that a good experimental design which has to be adopted for the optimization should employ the fewest number of measurements to get the greatest amount of information. In this light, the Taguchi experimental design has been very frequently used for screening the process variables that make the greatest impact on a process [19]. Accordingly, the present investigation has successfully employed the Taguchi experimental design to determine the relative importance of medium components and environmental factors in PHB production. Further, the variation of each factor was determined with orthogonal array. By studying the main effects of each of these factors, the general trend in the influence of factors towards the process can be characterized. Thus the characteristic of a particular influencing factor which could produce the preferred results can be controlled. Besides, the levels of factors which could produce the best results have also been predicted by Krishna Prasad *et al.* [20]. Dingra and Kumari Priya [21] have used different concentrations of jaggery (0.4 to 1%) and soybean (0.2 – 1%) flour as a carbon and nitrogen source

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respectively. They have found that the usage of 1% and 0.2 % soybean flour in minimal media as the most effective levels.

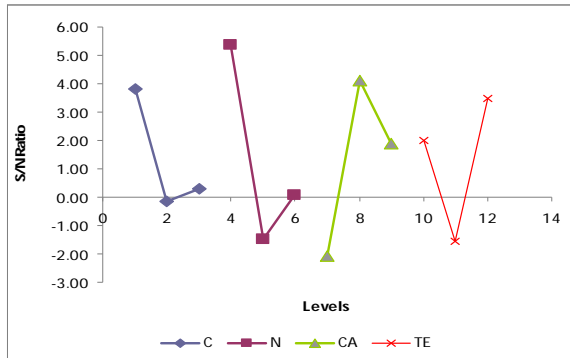


Figure 1: S/N ratio of biomass with various nutrients constitutes

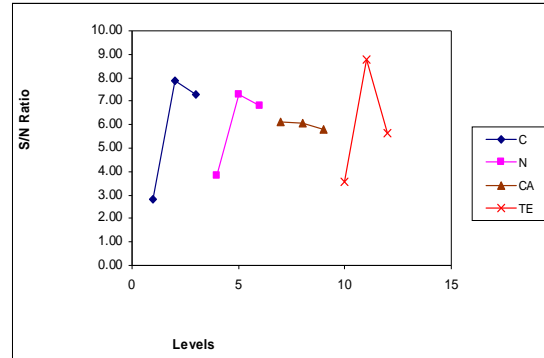


Figure 2 : S/N ratio of PHB in various nutrient constitutes

In this milieu, a wide variety of low cost carbon and nitrogen sources such as molasses and corn steep liquor [22], whey [23], banana pseudostem [24], damaged food grains, pea shells, apple pomace and palm oil effluent [25], starch [26] and dairy waste like cheese whey [27] have also been experimented by various researchers during the last three decades. As far as the present study is concerned, the carbon source was made available in excess and the nitrogen source in deficit. The nitrogen starvation condition is expected to generate high intracellular concentrations of PHB by slowing down the cell growth [28] [29] [30].

It is noteworthy that the synthesis of PHB can be correlated with the formation and consumption of acetate, an intermediate. The high concentration of PHB would suppress acetate formation. This is desirable because acetate inhibits cell growth [30]. On the other hand, the extremely fast synthesis of PHB seems to be unfavourable to cells, since it exerts high metabolic stress [31]. Therefore, the production of PHB and the level of acetate have to be balanced to optimize fermentation [32].

In the present investigation, the optimum nutrient condition for the growth media of the biomass were seemed to be 10 g/L - sago, 1 g/L - corn steep liquor, 2 ml/L - lemon juice and 30 ml/L tender coconut. On the other hand, for the production media of biopolymer, the optimum nutrient conditions opted were as follows: 20 g/L - sago, 2g/L - corn steep liquor, 3ml/L - lemon juice and 20 ml/L - tender coconut. Further, on subjecting the data obtained for Grey analysis against the various constituents / nutrients involved in PHB production (Table 4-6; Figure 1 -3) to ANOVA (Table 7), the results revealed their percentage contribution as 28, 23.6, 19 and 29 % against carbon, nitrogen, citric acid and trace elements respectively.

Table 7 : ANOVA for the nutrient sources and their % contribution

Parameters	DOF	SSQ	MEANSQ	F	% Contribution
C	2	0.050	0.025	60.372	28.072
N	2	0.042	0.021	50.818	23.629
CA	2	0.034	0.017	41.425	19.262
TE	2	0.052	0.026	62.950	29.270
Error	0	0.000			0.232
Total	8				100

Reema *et al.* [33], while assessing the effect of different carbon sources *viz.*, maltose, glucose, fructose, sucrose, lactose, arabinose and xylose on PHB production, have observed the maximum yield (49 mg/L) with maltose. Similarly, among different nitrogen sources (Ammonium Nitrate, Ammonium Citrate, Ammonium Sulphate, Glycine, Tryptone, Urea & Ammonium Dihydrogen Phosphate) tested against the PHB production, ammonium nitrate has been reported to show the highest (185 mg/L) yield [33].

Further, the effect of signal noise ratio (S / N ratio) value of optical density has also been worked out to determine the influence of different parameters on PHB production [17]. The effect of S / N ratio on optical density and the influence of different parameters on the biopolymer production are being shown in Table 8 - 11 and Figure 4 - 6. The optimum conditions for the production of biomass were found as pH 7, temperature 30 °C and 24 hrs of incubation period.

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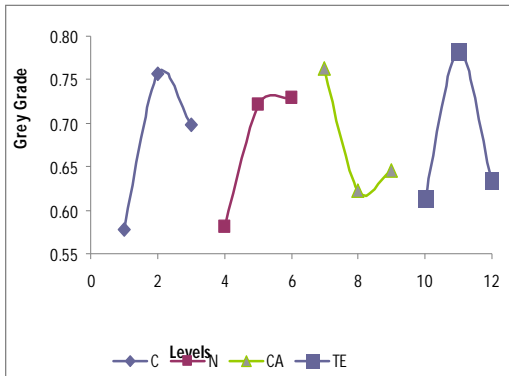


Figure 3 : Grey analysis for PHB production in various nutrient constituents

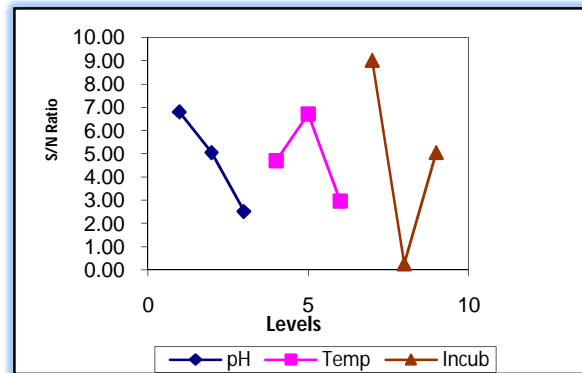


Figure 4 : S/N ratio of biomass against various physicochemical parameters

Wherein the biopolymer (PHB) production, except the incubation period (48 hrs), the optimal values of the other parameters (pH 7 & temperature 30 °C) remained same as in the earlier case. Vishnuvardhan Reddy *et al.* [34] have reported to show PHB production in 18 hrs and which reached the optimal level at 48 hrs in *Bacillus Spp.*

Table 8 : S/N ratio of PHB and biomass against physicochemical parameters

S.No.	pH	Temperature °C	Incubation Hrs	PHB g/L	S/N Ratio of PHB g/L	Biomass g/L DCW	S/N Ratio of Biomass g/L
1.	4	20	24	0.1	20.000	2.89	9.218
2.	4	30	48	0.632	3.986	1.62	4.190
3.	4	40	62	0.423	7.473	2.23	6.966
4.	7	20	48	0.119	18.489	1.283	2.165
5.	7	30	62	0.320	9.897	1.89	5.529
6.	7	40	24	1.529	-3.688	2.36	7.458
7.	9	20	68	0.536	5.417	1.368	2.722
8.	9	30	24	1.186	-1.482	3.325	10.436
9.	9	40	48	0.110	19.172	0.528	-5.547

Table 9 : Grading by Grey analysis with reference to S/N ratio for physicochemical parameters

S/N Ratio of Biopolymer	Normalize	Coefficient	S/N Ratio of Bio-Mass	Normalize	Coefficient	Grey Grade
20.000	0.000	0.471	9.218	0.924	0.921	0.696
3.986	0.676	0.733	4.190	0.347	0.577	0.655
7.473	0.529	0.654	6.966	0.783	0.804	0.729
18.489	0.064	0.487	2.165	0.482	0.632	0.560
9.897	0.427	0.608	5.529	0.693	0.744	0.676
-3.688	1.000	1.000	7.458	0.814	0.827	0.913
5.417	0.616	0.698	2.722	0.517	0.648	0.673
-1.482	0.907	0.905	10.436	1.000	1.000	0.953
19.172	0.844	0.851	-5.547	0.000	0.471	0.661

Table 10 : Mean response for the physicochemical parameters

Parameters	1-Mean	2-Mean	3-Mean
pH	0.693	0.716	0.762
Temperature	0.643	0.761	0.768
Incubation	0.854	0.625	0.693
Total	2.1904	2.1028	2.2228
Mean Grey	0.8145		

Table 11: ANOVA for the % contribution of the physicochemical parameters

Parameters	DOF	SSQ	MEAN SQ	F	% Contribution
pH	2	0.007	0.004	0.365	5.702
Temperature	2	0.030	0.015	1.455	22.719
Incubation	2	0.083	0.041	4.085	63.773
Error	2	0.010			7.806
Total	8				100.000

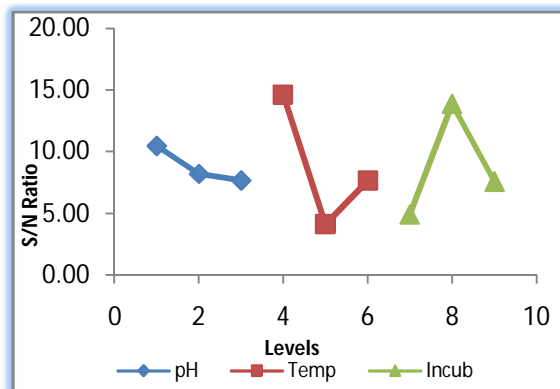


Figure 5 : S/N ratio of PHB against various physicochemical parameters

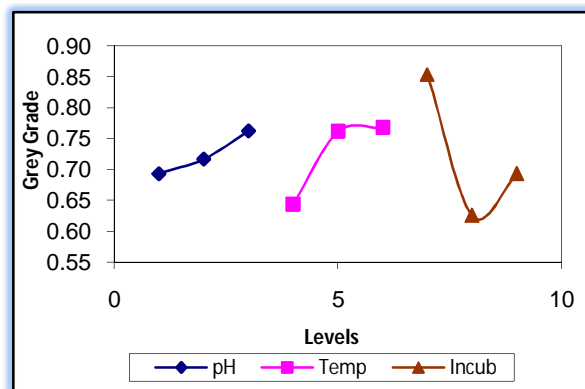


Figure 6: Grey analysis of PHB production against various physicochemical parameters

Fascinatingly, the very promising level of PHB (1.186 g / L) was recorded by the biomass of 3.325 g / L on dry cell weight basis, at 30 °C and 24 hrs of incubation (Table 21) . Followed by that, the next higher level of PHB (1.529 g / L) was recorded at 40 °C and 24 hrs of incubation with the biomass content, 2.36 g (dry cell weight) / L. Sangkharak and Prasertsan [35], while observing the three strains (ES16, N20 & U7) of halotolerant photosynthetic bacteria (*R. sphaeroides*) on cultivation in GM (Glutamic acid & Malate containing medium) with pH 7.0 , on a shaker (150 rpm) at 37°C for 96 hrs , recorded the PHB content of 4.44, 3.41 and 1.45 g / L against the biomass 8.23 , 8.12 and 7.44 g / L respectively. Whereas Hassan *et al.* [36] have reported 4 g/ L PHA with 67% DCW of *R. sphaeroides* IFO 12203, under anaerobic condition on 200 hrs cultivation.

With reference to the impact of pH on PHB production, in *E. aerogenes*, Reema *et al.* [33] have recorded the highest yield of 158 mg / L and 193 mg / L with pH 7 and 37 °C respectively. As for *E.aerogenes*, 48 hrs of incubation period is seemed to be optimum (185mg/L) for PHB production [33].

Contradictorily, Hamieh *et al.* [37] have reported with the maximum PHB production of 0.412 and 0.367g / 50 ml with *Lactobacillus acidophilus* and *B.thuringiensis* respectively at pH 5.5. Generally, the metabolic processes are highly susceptible to even slight changes in pH [38]. Besides, the bioavailability of trace elements would also be altered by pH of the medium [39] .

Yet another finding of Flora *et al.* [40] has revealed the maximum PHB production in *B.sphaericus* at the pH range 6.5 - 7.5. Nevertheless, Tripathi *et al.* [41] have reported 34 - 54 °C and pH 6.54 as optimal conditions for PHB production. However, at elevated levels of temperature (> 40 °C) and pH (>7.5) the PHB content seemed to be decreased with the enhanced PHB depolymerase activity [40] [41].

In the present investigation, while treating the Grey analysis data with ANOVA, the percentage contribution of the selected parameters such as pH, temperature and incubation period on PHB production were seemed to be 5.7, 22.7and 66.7 % respectively. Thus it is evident that the incubation time does play remarkably a significant role in PHB production.

It is believed that the cost of various ingredients of production media can have a profound impact on the overall cost of production and accounts for about 38 – 78% of the total production cost [42]. In general, the cost of the defined media (Basic Media) is seemed to be highly expensive when compared to the semi-defined medium. Further, the cost of the purified ingredients is also remarkably greater than the crude components.

Hence in the present investigation, with a view to design a cost effective media in an economically feasible manner, the ingredients were chosen and their cost was also worked out (Table 12). Accordingly, as on date, the cost of PHB

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production media of the present study is around ₹ 198 /- (€2.69; US \$ 2.93) per kg. This amount is seemed to be relatively cheaper when compared to the conventional routine chemical ingredients exploited in the production media, which amounted ₹ 319/- (€4.33; US \$ 4.71) per kg. However, this amount does not include manpower and the other operational costs.

Table 12: Cost of Raw materials used in PHB production

S.No.	Basic media		Cost effective media	
	Consumption	Cost (₹) of PHB Per Kg	Consumption	Cost (₹) of PHB Per Kg
1.	Glucose	144.00	Sago	40.00
2.	Ammonium nitrate	20.00	Corn powder	20.00
3.	Citric acid	20.00	Lemon Juice	15.00
4.	Trace elements	45.00	Tender Coconut	35.00
5.	MgSO ₄ .7H ₂ O	03.00	MgSO ₄ .7H ₂ O	03.00
6.	KH ₂ PO ₄	85.00	KH ₂ PO ₄	85.00
	Total	319.00	Total	198.00

On an average , even if US \$ 5 - 7 per kg is added to the substrate cost as the operational cost , the production cost of PHB would be ranging between US \$ 8 and 10 only. Chanprateep, [43] while reviewing the market price of PHAs for the year 2010, has shown an average of US \$ 3.7 / kg. To derive the current price, 2 % can be added per year to the cost of PHAs' 2010. Whereas Van - Thucoc *et al.*, [1] have worked out the production cost of commercially available PHAs as approximately US \$ 16 per kg. Thus in the present investigation, the production cost of PHB is seemed to be comparatively cheaper with the chosen substrates.

IV. CONCLUSION

In the present investigation, the routine NLMS medium has been made cost effective and thus proved to be economically viable. However, in near future, by genetically modifying the bacterial isolates of agro-industrial sites, for enhanced PHB production potentials, the production cost shall be reduced further. Besides, the performance properties and processability of PHB can also be improved with biobased additives. This practice would undoubtedly enable the biopolymers to compete with the existing non-renewable plastics. Thereby, assures green and clean environment.

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