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RESEARCH ARTICLE

Isolation, *Yield* optimization and Characterization of bioactive compounds from soil bacteria utilizing HPLC and Mass Spectra.

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Abstract:

The present study is an attempt to isolate and characterize the bioactive compounds produced by bacteria inhabiting in the soil together with the optimization of yield of the compounds. For the purpose, soil samples from different locations were collected and screened for isolation of soil bacteria. The property of the bacteria to secrete bioactive compounds was tested by observing the zone of inhibition against *Staphylococcus aureus,* an indicator organism. Morphological identification of these bacteria has been carried out. Furthermore, a suitable method of optimization was applied to improve the productivity of bioactive substances and the productivity has been statistically validated using Taguchi methods. Preliminary isolation of these bioactive substances is carried out on the basis of their solubility characteristics. Further extensive isolation and partial characterization of these bioactive substances were performed using RP-HPLC. The isolated bioactive compounds were further tested by mass Spectra for determination of their purity and molecular weight.

Keywords: *Soil bacteria*, bioactive compounds, Gel electrophoresis, Taguchi design, RP-HPLC, Mass Spectra.

Introduction:

The natural sources of bioactive compounds are bacteria, fungi, actinomycetes, algae, lichen and green plants. The importance of Actinomycetes has received limelight attention after the discovery of actinomycin in 1940 and streptomycin in 1944 by Waksman [1-3]. The genus Streptomyces was proposed by Waksman and Henrici [4] for aerobic, spore forming Actinomycetes. It has been found out that members of Streptomyces are a rich source of bioactive compounds like antibiotics, enzyme inhibitors and pharmacologically active agents [5-10]. Almost 75% of known commercially useful antibiotics are produced by soil bacteria such as Streptomyces [11-13]. Though Waksman pointed out that the natural substrates are ideal sources for isolation of Actinomycetes, common sources are generally soil water and air [14]. Furthermore it has been estimated by Beijerinck [15] that Actinomycetes are found in great abundance in soil. The soil bacteria producing antibiotics have been screening for the last few years at a very rapid pace. Because of resistance to bacterial strain and side effects to a great extent, a considerable amount of research have been carried out to develop new antibiotics are validated by using Taguchi methods, a sophisticated statistical tool. Bioactive compounds in the biological sample have been separated by RP- HPLC using C_{18} having potential therapeutic effect with minimum side effect. Moreover the assay of a new one can be easily performed by clinical microbiologists. To discover new antibiotics, it is necessary to continue the use of conventional screening programs. An attempt has been made to perform intensive screening programs on different soil samples collected from remote areas of Salt lake Chilika in Orissa and hence likely to yield purposeful results towards isolation of species of soil bacteria which may produce promising bioactive compounds. The morphological properties of these bacteria have been identified and characterized by compound microscope and their DNA bands are observed in a gel electrophoresis. The identification of natural product traditionally involves the scale up of fermentation broth. An experimental design has been carried out to develop a statistical model for better understanding the influences of biophysical factors such as nutrient concentration, temperature, salt concentration and pH which affect the rate of productivity. From these models the relative influences of various factors at different level and their interaction can be determined. The optimal level of bioactive production is calculated by observing the enhancement of zone of inhibition against *Staphylococcus aureus* and the results column. The antibiosis property of the isolated compounds has been tested. The molecular weight and

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fragmentation pattern of these compounds were performed by mass spectra.

Materials and Methods:

Isolation of bacteria from soil sample:

In a systematic screening program for isolation of bioactive compounds from soil bacteria, a total number of four different soil samples were taken in sterile tubes using a sterile spatula. It should be noted that the points of collection is as widely varying characteristics as possible with respect to the organic matter, moisture content, particle size and color of soil.

A number of five 150 ml conical flasks are taken, each of which contains 1 gm of soil sample mixed with 100 ml sterile distilled water, Phosphate buffer (0.13M NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4, pH-7.2), 0.9% saline solution, 6.5 % NaCl solution, respectively and each flask is stirred by a teflon coated magnet bar for 30 minutes to detach the spore chains if any. 1 ml of sample solution is taken from each flask and transferred into two 2 ml of sterile ependroff tubes. Then 1 ml of aliquot from each ependroff tube is added to 9 ml diluent nutrient broth in a borosilicate glass and capped it tightly. Sonication is performed for one of the tubes at 20 KHz frequency and intensity of 2 W/cm^2 for 30 minutes to increase growth rate by effective transport of oxygen and nutrient in suspension. The other tube is mixed with a vertex mixture at 150 rpm for 15 minutes followed by 48 hour incubation [16, 17]. Then1 ml of sample from each tube is made to spread over a freshly prepared agar plate with nutrient broth and is kept for 48 – 72 hours to visualize the growth of colonies. The same process is repeated for all the flask contents by using LB broth. The above two methods are repeated by mixing soil extract found in its native environment, the procedure of which is given below [18].

10 gm of soil was added to 100 ml of tap water and autoclaved at 121°C for 20 minutes. The sample was filtered and volume was restored to 100 ml using tap water. 1.5% agar and 0.02 gm K_2HPO_4 was added to it and again autoclaved at 121°C for 20 minutes. Soil sample is added through serial dilution method with 10 fold decrease in concentration in each corresponding tube for isolating the colonies until the number of colonies formed up to 20 to 200 for a valid counting. To grow certain new bacteria, 10% soil extract is mixed with 1% yeast extract followed by 1.5% agar and autoclaved at 121°C for 20 minutes. Then soil sample is added through serial dilution method with 10 fold decrease in concentration in each corresponding tube for isolating the colonies. Then morphological identification was carried out to characterize number of different colonies.

Identification and characterization of bacteria producing antibiosis:

A number of 12 bacterial strains named as ICB-0011, ICB-0012,……ICB-0022 were identified based on their morphological characters such as size, shape, color, texture etc. using compound microscope. Details of morphological characters of bacterial strains are shown in Table.1

. Strain of bacteria	Color	Elevation	Sheen	Margin	Size	Melanoid pigment	Growth Pattern(Before & after optimization)
ICB-oo11	Yellowish white	Flat	shining	Circular	Spreading	-	----
ICB-oo12	Yellowish white	Flat	shining	Circular	spreading	-	-----
ICB-oo13	Brownish white	Raised	smooth	undulated	small	-	-----
ICB-oo14	Brownish white	Flat	smooth	Lobate	small	-	----
ICB-oo15	Creamy white	Convex	shining	Circular	spreading	-	SAME (as typical)
ICB-oo16	Yellow	Flat	smooth	Lobate	punctate	-	CHANGES(in log phase)
ICB-oo17	yellow	Raised	dull	undulated	small	-	----
ICB-oo18	Translucent	Umbonate	dull	Rhizoid type	large	-	SAME(as typical)
ICB-oo19	Creamy translucent	Raised	dull	Circular	large	+	SAME(as typical)
ICB-oo20	Reddish white	Convex	shining	undulated	small	+	------
ICB-oo21	Reddish white	Flat	smooth	undulated	spreading	-	------
ICB-oo22	white	Raised	smooth	Circular	small	-	--------

Table 1: Morphological characterization of isolated 12 bacteria

These bacteria were again tested for their capability of producing antibiosis. It is performed by preparing assay plates with 25 ml of nutrient agar and considering *Staphylococcus aureus* as test organism. Agar cups are filled with 5 ml of mycelia free culture filtrate in triplicate and the plates were incubated at 37^0c for 24 hour to measure the area of zone of inhibition. It has been found that four of them mentioned as ICB-0015, ICB-0017, ICB-0018, ICB-0019 shows good inhibitory activity in mycelia free broth culture extract. Therefore, these four types of bacteria are further identified according to size of DNA bands in a gel electrophoresis (Figure 1). These bands indicate the separation of genomic DNA of these four bacteria and hence they are preserved for detection of a specific DNA sequences in these DNA samples for future identification from various sources and to observe the presence of ppGpp sequences, a conserved sequences in antibiotic secreting bacteria which is beyond the scope of this present work [19].

Figure 1: DNA bands in gel of four isolated bacteria having antibiosis activities in broth culture.

Results and Discussion:

Optimization Study to Increase Bioactive Yields:

Optimization is performed to improve the yield of bioactive substances produced by bacterial strains. From the zone of inhibition, it is observed that ICB-0015 gives much greater inhibitory activities than Therefore, an attempt has been made to improve the yields of ICB-0015 by optimizing biophysical parameters such as temperature, pH, salt and nutrient concentrations considering at three levels of experiments. Taguchi's experimental design has been applied to find the optimum combination of these four factors for maximum yield. Each factor is taken at three levels in an L_9 orthogonal array and experiment is performed in triplicate and the average area of zone of inhibition is calculated [20]. The structure of Taguchi's orthogonal robust design and the measurement of different parameters are being shown in Table 2.

Designation	Parameters	Level 1	Level 2	Level 3
A	Nutrient Conc.	0.1	0.2	0.5
B	Salt Conc.	0.1	0.2	0.5
C	Temperature	16	26	38
D	pH	6.5	7.0	7.5

Table 2: Different parameters considered in the Taguchi design at three levels.

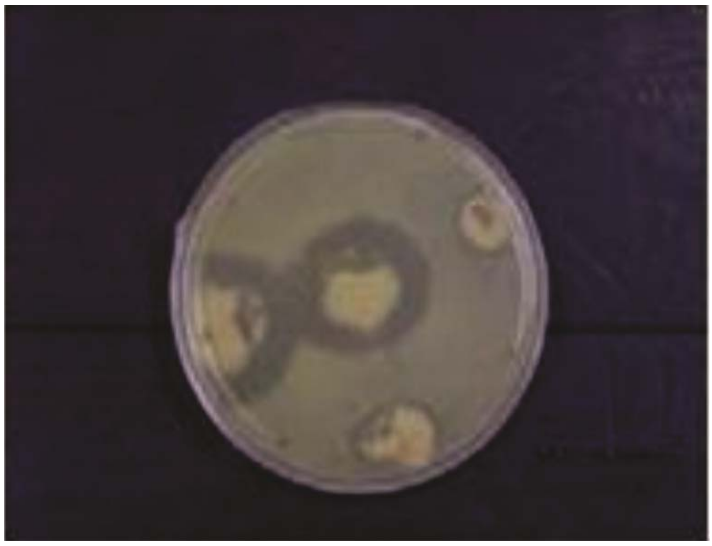

Figure 2: Zone of inhibition produced by ICB-0015 bacteria against *Staphylococcus aurous.*

In this work, we have taken nutrient concentration at three level of 0.5ml, 0.2 ml, 0.1ml respectively of 1X nutrient broth, salt (NaCl) concentration of 0.1N, 0.2N, 0.5N respectively, temperature of 16, 26, 38 degree Celsius respectively and pH at 6.5, 7.0, 7.5 respectively.

From the experiment, it has been found that the highest zone of inhibition area ($972mm^2$) was shown at a combination of 0.5ml nutrient broth, 0.5N salt concentration, temperature 26^0 C and pH 6.5. The values of S/N ratio of Taguchi method are being used to measure the characteristic deviating from the desired inhibition zone. The S/N ratios may differ according to the characteristics of environmental condition. In that case the bigger characteristics (ZoI) are better; the S/N ratio is defined as

$$S/N \text{ ratio [dB]} = -10 \log[(1/y_1^2 + 1/y_2^2 + 1/y_3^2 + \cdots)/n]$$

Where, y_i is the characteristic property (zone of inhibition) and n is the replication number of the experiment [21]. The mean S/N ratio for each level of the parameters is summarized and the S/N response table for inhibition area and standard deviation is shown in Table 3. The zone of inhibition should remain high as most of the experiment produce zone of inhibition greater than a minimum critical value. Zone of inhibition of bioactive compounds

produced by ICB-0015 during the optimization study is given in Figure 3.

Exp. No	A	B	C	D	Average ZOI	S/N ratio
1	0.1	0.1	16	6.5	825	58.3291
2	0.1	0.2	26	7.0	813	58.2018
3	0.1	0.5	38	7.5	642	56.1507
4	0.2	0.1	26	7.5	713	57.0618
5	0.2	0.2	38	6.5	916	59.2379
6	0.2	0.5	16	7.0	785	57.8974
7	0.5	0.1	38	7.0	891	58.9976
8	0.5	0.2	16	7.5	853	58.6190
9	0.5	0.5	26	6.5	972	59.7533

Table 3: Experimentally measured value and S/N ratio for Zone of Inhibition

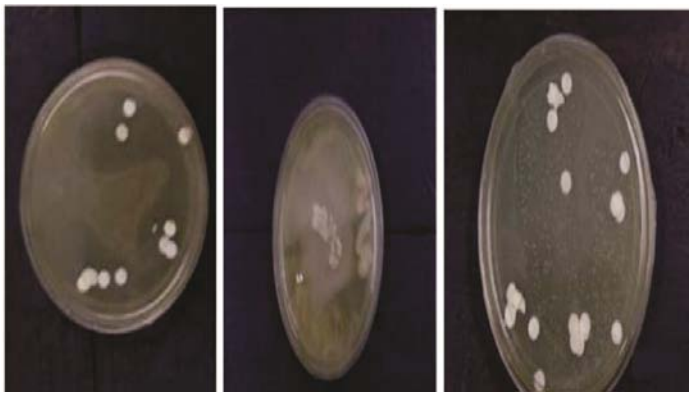

Figure 3: Zone of inhibition of bioactive compounds produced by ICB-0015 during the optimization study

Theoretical prediction of optimization for bioactive yields:

The optimal parameters have been predicted theoretically using Taguchi model. The predicted optimal levels of factors are nutrient concentration at 0.5 ml, salt concentration at 0.2ml, temperature at 26^0C and pH at 6.5 which is given in Figure 4.

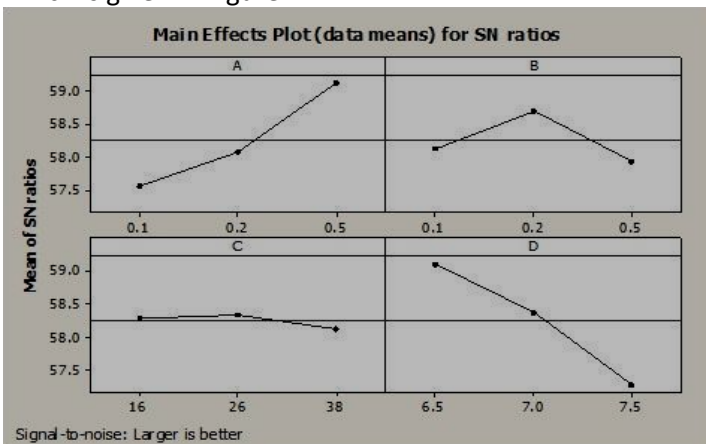

Figure 4: Theoretical prediction of optimal parameters using S/N ratio.

The predicted S/N ratio using the optimal level of design parameters can be calculated by $S/N_{predicted} = S/N_m + \{(S/N_3 - S/N_m) + (S/N_2 - S/N_m) + (S/N_2 - S/N_m) + (S/N_1 - S/N_m)\}$

Where, S/N_m the total mean S/N ratio and S/N_3 S/N_2 S/N_2, SN_1 is the mean S/N ratio at the optimal level [21]. In the case of zone of inhibition, the mean value calculated from table 3 is about 58.24. Also the S/N ratio for A3, B2, C2 and D1 can be obtained from Table 4 and the values are 59.11, 58.68, 58.33 and 59.1 respectively.

Parameters	S/N ratio at three levels		
	Level 1	Level 2	Level 3
A	57.56	58.06	59.11
B	58.12	58.68	57.93
C	58.27	58.33	58.12
D	59.11	58.36	57.27

Table 4: S/N ratio for zone of inhibition

By using the above equation the predicted S/N value is $S/N_{predicted} = 58.24 + (59.11-58.24) + (58.68-58.24) + (58.33-58.24) + (59.1-58.24)$, so that the predicted value is 60.5. The value of zone of inhibition can be obtained using the following formula

$60.5 = -10 \text{ Log } (1/y^2)$

The estimated zone of inhibition is about 1059.2 mm^2. Table 5 shows the comparison of predicted zone of inhibition with the experimental results which shows some improvement in zone of inhibition. Hence the estimated the predicted data and experimental data are almost consistent with each other.

	Level	Area of zone of inhibition in mm^2	S/N ratio
Prediction	A3B2C2D1	1059	60.5
Experiment	A3B2C2D1	1013	60.11

Table 5: Results of the confirmation of the experiments for Zone of inhibition

Analysis of the bioactive yield using HPLC and Mass Spectra:

The bacterial free filtrate of optimized broth culture is obtained through filtration using sterilized Whatmann filter paper under aseptic condition. 50 ml of the filtrate was freeze-dried to obtain a highly viscous product. These products were then subjected for separation of ether soluble extractives, methanol soluble extractives and organic solvent insoluble extractives respectively. These organic solvent soluble solid products were separated by evaporation through a rotary vacuum evaporator. The solid products thus obtained from the above organic solvent were dissolved in 0.2 ml of water and 50 micro liter of the solution is applied on assay plates to test the fraction showing zone of inhibition [22,23]. It has been observed that only methanol soluble extractive

possess antibiotic activity against *Staphylococcus aureus* and retains the activity up to 60 times of dilution with millipore water. Figure 5 shows zone of inhibition produced by methanol soluble extractive. For isolation of the antibiotic from methanol soluble extractive, HPLC analysis has been performed. 100 micro liter sample of the methanol soluble extractive solution is diluted 20 times for producing effective separation.

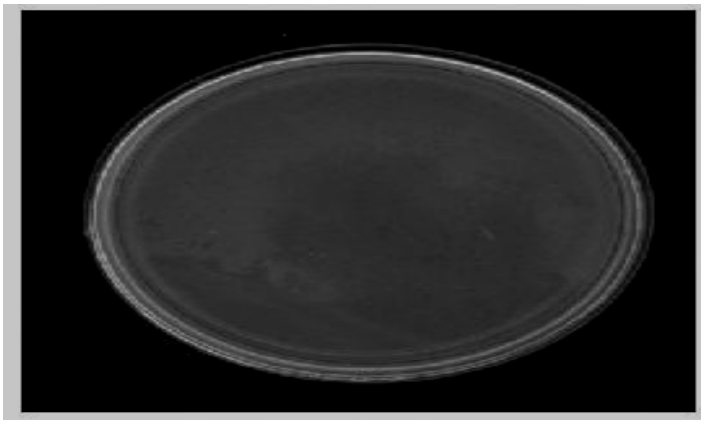

Figure 5: Figure 5 shows zone of inhibition produced by methanol soluble extractive obtained from ICB-0015.

. UV–Visible spectra of this sample show the two prominent peaks of absorbance at a wavelength of 260 and 280 nm. So these two wavelengths have been set in the PDA detector of HPLC. The parameters such as temperature, composition of mobile phase, pH, flow rate, isocratic and gradient elution mode are optimized to find out chromatogram having well resolution to obtain the compound showing antibiotic activity. Different fractions of samples are being collected corresponding to different number of peaks in the chromatogram of HPLC in a 10 ml vial and designated with a number corresponds to the peaks. The HPLC is allowed to run a number of times to collect about 6 to 7 ml of each fraction of sample so as to obtain the concentration of the sample solution above minimum inhibitory concentration. They are then again Freeze dried to isolate the separated product and tested for antibiotic activity by observing zone of inhibition. It has been found out that the fraction corresponds to peak-1 shows antibiotic activity. Then this fraction of product has been analyzed through mass spectroscopy in positive ion ESI mode to obtain the molecular weight of the compound [24-28]. For effective separation of bioactive compounds from the methanol soluble extract of ICB-0015, 0.1% Trifluroacetic acid, 10% methanol and HPLC grade water were taken as mobile phase composition. The pH should be 7.0 and flow rate is 0.5 ml/min. It has also been observed that using acetonitrile instead of methanol as a mobile phase gives a number of small peaks those were absent in very first time run of the sample but no such peaks were observed while using methanol. Moreover, the freeze dried product of the fraction showing antibiosis is less as compared to the mobile phase containing methanol mixed with water and TFA. The reason behind the running of sample at 5 to 6 times is that the collection of a considerable amount of inhibitory concentration. Hence, it has been suggested

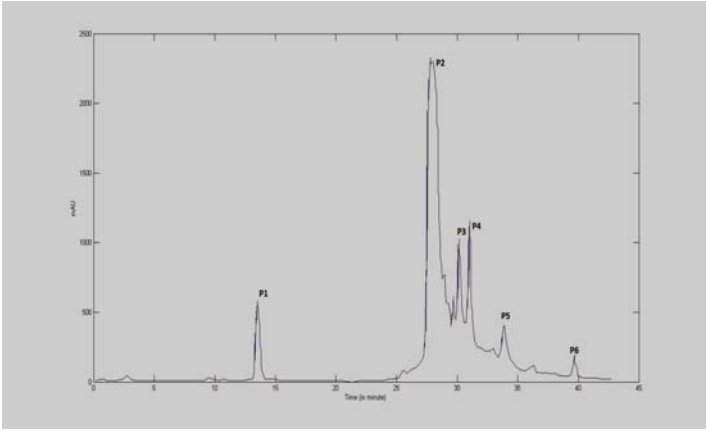

Figure 6: Chromatogram of methanol soluble extractive

that the bioactive compound in the mixture may get oxidized in presence of acetonitrile. It has been found that with low percentage of methanol, a stable signal of chromatogram was not found. So varying the concentration of methanol and acetic acid can give a clear impact on both the intensity and charge envelop. The effective separation has occurred at a concentration as per the data mentioned above. Figure 6 signifies chromatogram of the fraction of methanol soluble extractive of bioactive compound produced from ICB-0015. The fraction of bioactive component corresponds to peak P1 of chromatogram (Figure 6) shows antibiotic activity and is being named as GRNP-1. Finally, mass spectroscopic analysis has been performed for this compound to obtain the Molecular weight and fragmentation pattern as shown in Figure 7. The different peaks obtained with m/z (mass/charge) ratio for GRNP-1 from right to left are 896, 895, 516, 478, 477, 476, 475, 461, 460, 438, 367, 307 and 254.

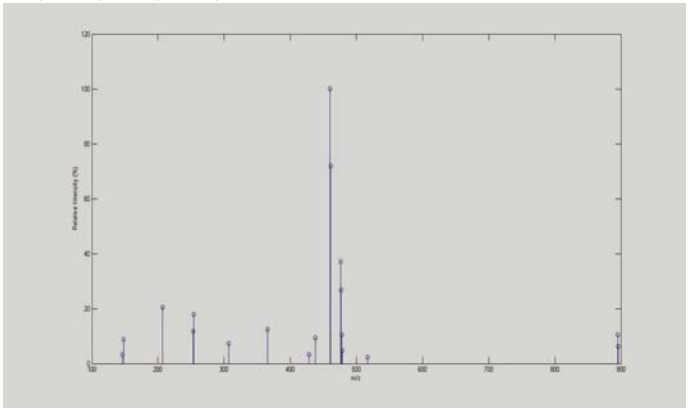

Figure 7: Mass spectra of fraction corresponds to peak P1 of GRNP 1

The interpretation is as follows: 437+23=460 i.e. M.W + Na, [(437*2)+23]-1=2M^++ Na^+ - H^+= 896, 516 - 477=39 (K)437+1=438 i.e M+1 peak, 516-437=79 (DMSO+H)

Hence, the result confirms that molecular weight (M.W.) of the compound GRNP-1 is 437 which shows highest zone of inhibitory activity. Results reveal that GRNP-1 consists of compound having molecular weight of 437 which may exist as dimer as evident from its Mass spectra.

CONCLUSION:

In the present study, isolation, improvement of yield and characterization of bioactive compounds from soil bacteria has been carried out. It was observed that many of the soil bacteria secrete bioactive substances only when they present in group or cluster. An attempt has been made to improve the production and leaching rate of bio active compounds in a broth culture by using suitable parameters such as pH, salt concentration, nutrient amount and temperature which regulate bio physical property of the organism for antibiotic production. Taguchi's parameter design and optimization has been found successful in improving the yield of bioactive substances. It has shown that separation of bio active compounds from bacteria through RP-HPLC depends on polarity and pH of the mobile phase as these two factors affect the distribution of charge and hence the affinity towards C_{18} column. Gradient analysis mode in some biological sample may provide better resolution of peak, but in our case it lacks any significant improvement. Mass Spectra analysis gives molecular weight of 437 of the most bioactive compound named as GRNP-1. Hence, the present study could provide an idea for future analysis of structure elucidation of this highly bioactive compound with antibiosis activity and development of QSAR models through structure-property-activity relationships.

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