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CHEMICAL EXTRACTION AND OPTIMIZATION OF INTRACELLULAR β-GALACTOSIDASE PRODUCTION FROM THE BACTERIUM *ARTHROBACTER OXYDANS* USING BOX-BEHNKEN DESIGN OF RESPONSE SURFACE METHODOLOGY

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Present study demonstrated the isolation of most promising β-galactosidase producing bacterial strain SB from soil. Morphological, biochemical, and 16s rRNA sequence analysis identified the bacterial strain as *Arthrobacter oxydans*. Several chemicals, including SDS, Triton X-100, Tween 20, isoamyl alcohol, and toluene-acetone mixture, were applied for extraction of intracellular β-galactosidase from the bacterial strain *Arthrobacter oxydans*. Among these, Tween 20 was recorded to be most effective. Role of pH, temperature, and shaker speed on production of β-galactosidase was evaluated using Box-Behnken design of response surface methodology. According to Box-Behnken analysis, optimum production of β-galactosidase (21.38 U (mg^{-1} protein)) is predicted at pH 6.76, temperature 36.1 °C, and shaker speed 121.37 r.p.m. The parameters are validated with the nearest value.

Keywords: β-galactosidase, *Arthrobacter oxydans*, chemical extraction of β-galactosidase, optimization, response surface methodology

β-Galactosidase is an enzyme that hydrolyses the complex lactose into simple sugars, glucose, and galactose. Among different functions, two most important roles of β-galactosidase are removal of lactose from milk for lactose intolerable people and glycosylated product formation (Hsu et al., 2005; Heyman, 2006). β-galactosidase is a commercially important enzyme that are widely used in different industries, especially in food and pharmaceutical sectors. (Pierre, 2004; Hartmann, 2005; Husain, 2010). According to the existing reports, major β-galactosidase producing bacteria species belong to *Bacillus subtilis* (El-Kader et al., 2012), *Lactobacillus gasseri* (Honda et al., 2012), *Streptococcus thermophilus* (Princely et al., 2013) and *Anoxybacillus* sp. (Osiriphun & Jaturapiree, 2009).

β-Galactosidase produced by bacteria may be extracellular or intracellular. Prasad and co-workers (2013) reported the characterization of extracellular β-galactosidase produced by *Bifidobacterium animalis* spp. Whereas, Maity and co-workers (2013) characterized both extracellular and intracellular β-galactosidase from two bacterial strains BS1 and BS2. Isolation of intracellular β-galactosidase is harder and greatly varies from one bacterial species to another. Several methods have been implemented for isolation of intracellular β-galactosidase. There are few reports regarding the permeabilization of bacterial cells for release of intracellular β-galactosidase with the help of organic chemicals such as chloroform,

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toluene, and ethanol (Numanoglu & Sungur, 2004; Panesar et al., 2007; Park et al., 2007). Beside organic solvent, many researchers also applied different mechanical methods for isolation of bacterial intracellular β-galactosidase. Among these, sonication and high pressure homogenization were reported to be most effective for isolation of intracellular β-galactosidase (Geciova et al., 2000; Osiriphun & Jaturapiree, 2009; Saishin et al., 2010).

Production of enzyme largely depends on medium composition and different physical factors like pH, temperature, incubation period, inoculum size, carbon and nitrogen sources (Ray et al., 2012). Regardless of the other process, it is necessary to monitor and control parameters like moisture content, temperature, pH, incubation period, inoculum size, and selection of optimum carbon and nitrogen sources (Ray et al., 2007). Therefore, our aims of this study are (i) isolation, characterization, and identification of β-galactosidase producing bacterial strains, (ii) extraction of intracellular β-galactosidase from the selected bacterial strain by different chemical reagent, and (iii) optimization of culturing parameters for better β-galactosidase production using response surface methodology (RSM).

1. Materials and methods

1.1. Screening and isolation of β-galactosidase producing bacteria

Soil samples were collected from different parts of Noida, Uttar Pradesh, India. One gram of soil sample was suspended in 10 ml of phosphate buffer (50 mM, pH 7.0) and serial dilution was made following the method of Ray and co-workers (2007). Separated bacterial colony from TSA medium were further screened for β-galactosidase producing on lactose agar plates (0.5% lactose, 0.5% peptone, 0.3% beef extract, agar 1.5%, pH 7.0) containing X gal (0.2 mg ml^{-1}) and incubated for 24 h at 37 °C. Appearance of distinct blue colour colonies indicates the production of β-galactosidase. Pure culture was made by repeated streaking method.

1.2. Phenotypic characterization of the bacterial strains

The bacterial strain, exhibiting the highest β-galactosidase activity, was primarily identified by morphological, physiological, and biochemical characteristics. Gram's staining was done according to the standard method of Christian Gram. Carbohydrate utilization test of the selected bacterial isolate was performed using HiMedia biochemical kits (KB009A and KB009B1).

1.3. Identification of the bacterial strains by 16S rDNA sequence analysis

Finally the bacterial strain SB1 was identified by 16S rDNA sequence analysis. Genomic DNA was isolated by traditional method using lysozyme and SDS. PCR amplification was done using 27f (AGAGTTTGATCMTGGCTCAG) and 1327r (CTAGCGATTCCGACTTCA) universal primers. Nucleotide sequence obtained was submitted to GeneBank for accession number. Phylogeny was drawn using Mega 4 software.

1.4. Extraction of intracellular β-galactosidase

Isolation of intracellular β-galactosidase from the selected bacterial strain was done by different methods.

1.4.1. SDS method. Fourteen-hour culture of the bacterial strain was used. Cells were collected by centrifugation (8000 r.p.m., 10 min) at 4 °C. Bacterial cells were resuspended in 500 µl of Z buffer (pH 7.0) containing 50 µl of 1% SDS, and incubated overnight (37 °C). The supernatant was collected by centrifugation (8000 r.p.m., 10 min) at 4 °C and used for enzyme assay.

1.4.2. Triton X-100. Cell pellet collected by centrifugation was resuspended in 500 µl of Z buffer (pH 7.0) containing 50 µl of 1% Triton X-100 and incubated overnight. The supernatant containing the crude enzyme was used for enzyme assay.

1.4.3. Toluene-acetone mixture. The bacterial cell pellet obtained by centrifugation was resuspended in 500 µl of Z buffer (pH 7.0). Fifty microlitres of toluene-acetone mixture (9:1) was added and kept for overnight. The mixture was centrifuged and the supernatant collected was used for β-galactosidase assay.

1.4.4. Tween 20. Cell pellet collected by centrifugation was suspended in 500 µl of Z buffer (pH 7.0) containing 50 µl 1% of Tween 20 and incubated for overnight. The supernatant collected by centrifugation was used for enzyme assay.

1.4.5. Isoamyl alcohol. Cell suspension in Z buffer (pH 7.0) was treated with 100 µl of isoamyl alcohol and kept for overnight. The supernatant containing the crude enzyme was used for enzyme assay.

1.5. Enzyme assay

Standard assay of β-galactosidase was performed using ONPG as a substrate in 50 mM Z buffer (pH 7.0) following the method of Choonia and Lele (2013) with few modifications. The activity of β-galactosidase was measured by incubating 30 µl of supernatant containing crude enzyme, 160 µl of substrate (4 mg ml^{-1}), and 0.7 ml of Z buffer at 37 °C for 30 min. Reaction was terminated by adding 0.4 ml sodium carbonate (1 M). The concentration of *ortho*-nitro phenol (ONP) was determined by spectrophotometry at 420 nm. Protein content was measured according to Lowry and co-workers (1951).

1.6. Culture medium and parameters

Production of β-galactosidase was carried out in 500 ml Erlenmeyer flask containing 250 ml lactose broth (g l^{-1} lactose, 10; beef extract, 15; peptone, 5; yeast extract, 0.5; NaCl, 1.5). Optimization of β-galactosidase production was done at different pH, temperature, and shaker speed.

1.7. Experimental design and statistical analysis

Three variables (pH, temperature, and shaker speed) Box-Behnken design for response surface methodology was carried out to optimize the β-galactosidase production. Statistical analysis was done using Minitab 17 software. Level of different factors was taken as –1, 0, and +1 (Table 1). Fifteen experiments were conducted to maximize the effect of unexplained

variability using specific β-galactosidase activity as response. Relationship between coded value and actual value in this experiment was given in Equation 1.

$$X_i=x_i-x_0/\Delta x;\ i=1,2,3 \quad (1)$$

where x_i denotes coded value, x_0 denotes actual value, and Δx denotes step change of x_i. Optimum conditions for β-galactosidase production were predicted from a second order polynomial model as described below in Equation 2.

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2 \quad (2)$$

where Y denotes response, b_0 denotes model constant, X_1, X_2, and X_3 were the variable, b_1,b_2, and b_3 are linear interaction, b_{12}, b_{13}, and b_{23} are cross product interaction, b_{11}, b_{22}, and b_{33} are quadratic interaction.

Table 1. Morphological and biochemical characteristics of the selected strain SB

Parameter	Bacterial strain SB
Morphology	
Pigment	Creamy white
Surface	Smooth
Margin	Regular
Elevation	Convex
Gram stain	+
Biochemical	
Citrate	+
Methyl red	–
Voges-Proskauer	–
Indole	+
Catalase	+
Urease	+
Gelatine	+
Glucose	+
Fructose	+
Maltose	–

+: positive; – negative

2. Results and discussion

The most promising β-galactosidase producing bacterial strain SB was identified by morphological, as well as biochemical characterization (Table 2). It was detected that the bacterial strain is able to produce indole, catalase, urease, as well as it can actively utilize glucose, fructose, and gelatine as their carbon source. Finally, the bacterial strain SB was identified by 16S rRNA sequence analysis (Fig. 1). Based on nucleotide sequence homology, the bacterial strain SB was identified as *Arthrobacter oxydans* (GenBank Acc. No. KM402760). β-Galactosidase is considered to be one of the most important enzymes that is widely used in different industries (Pierre, 2004; Panesar et al., 2006). It can be found in different groups of microorganisms, plants, and animals, which differ greatly in their properties and structure (Panesar et al., 2010). Production of β-galactosidase from plant and animal sources is not commercially significant due to higher cost and less yield. Therefore, screening of β-galactosidase producing bacteria is essential for industrial use.

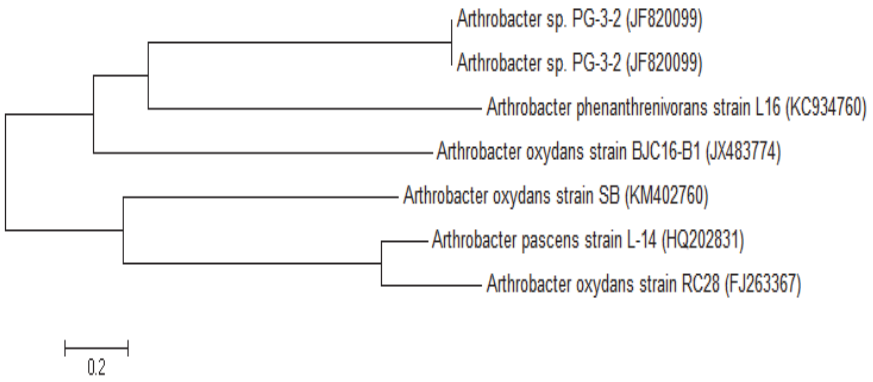

Fig. 1. Phylogenetic relationship of the bacterial strain *Arthrobacter oxydans* SB with other close homologous strains

Table 2. Factors used for experimental design

Factor	Name	Level		
		–1	0	+1
X_1	pH	6.0	7.5	9.0
X_2	Shaker speed (r.p.m.)	100	150	200
X_3	Temperature (°C)	25	35	45

Extraction of intracellular β-galactosidase is quite difficult and need proper standardization. In this present investigation, different types of cell wall disrupting chemicals were used (Fig. 2). Treatment with SDS and Triton X-100 does not show any significant difference as compared to the cells sonicated alone. On the other hand, toluene-acetone mixture, Tween 20, and isoamyl alcohol demonstrate remarkable increase in β-galactosidase recovery with the maximum amount in Tween 20 treatment. Bury and co-workers (2001) stated that sonication and French pressure was not suitable for large scale production of β-galactosidase from bacterial strain *Lactobacillus delbrueckii.* Whereas, Feliu and co-workers (1998) reported high β-galactosidase production from the bacterial isolate *Escherichia coli* using ultrasonication. Our experiment demonstrated that Tween 20 was the most effective chemical for extraction of β-galactosidase from the bacterial strain *Arthrobacter oxydans* SB, followed by toluene-acetone mixture and isoamyl alcohol, respectively.

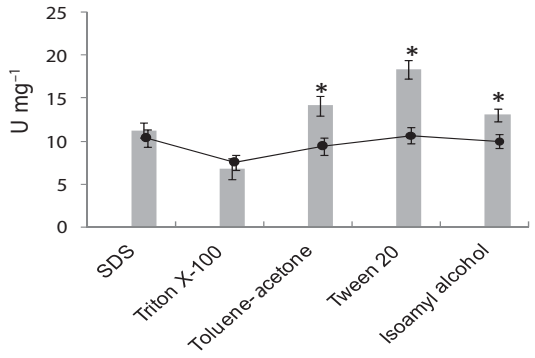

Fig. 2. Extraction of β-galactosidase form bacterial strain *Arthrobacter oxydans* SB using different chemical methods considering sonication as control. U=μg of product liberated /min (*: P<0.05).
▇: different methods of chemical extraction; —●—: sonication (control)

In this present investigation, Box-Behnken design was used and experimental data of 15 runs were summarized in Table 3, which indicated the interaction between different variables (temperature, pH, and shaker speed). According to the results obtained from the analysis of variance, a second degree polynomial model was fitted to this present experiment (Table 3). The P value for lack of fit (P>F) is 0.136, suggesting that this model adequately fits the data and there is only 13.6% chance that it is due to noise. The "lack of fit" F value is 6.51, which indicated an insignificant lack of fit. The model F value of 13.09 and low probability value imply the significant model fit (Table 4). The coefficient of determinant (R^2) was 0.9593, which indicated the variability of the model, as well as real relationship between variables. This R^2 value explained the variability of the model by 95.93% and only 4.07% by chance. The coefficient of variance (C.V.) of the model was 5.06%, which indicated the degree of precision. The adequate precision value was 9.957, which simply indicated the signal to noise ratio (Table 5). According to Cao and co-workers (2009), the desired adequate precision value must be greater than 4 for reliability of model. Multiple regression analysis of the experimental data, followed by second degree polynomial equation (3) was found to describe the interaction between pH, temperature, and shaker speed for β-galactosidase production.

$$\text{Specific activity} = -95.2+28.93\text{pH}+0.787\text{Temp}-0.0685\text{Speed}-1.902\text{pH}\times\text{pH}-0.00874\text{Temp}\times\text{Temp}+0.000143\text{Speed}\times\text{Speed}-0.0317\text{pH}\times\text{Temp}+0.00155\text{pH}\times\text{Speed}+0.000456\text{Temp}\times\text{Speed} \quad (3)$$

where pH, temperature, and speed are the three variables already mentioned in Table 1.

Table 3. Box-Behnken experimental design showing β –galactosidase activity at different variable combinations

Run	Coded value			Actual value			β-galactosidase activity (U (mg protein^{-1}))
	X_1	X_2	X_3	pH	Shaker speed	Temperature	
1	–1	0	+1	6.00	150.00	45.00	15.01
2	0	0	0	7.50	150.00	35.00	22.19
3	–1	+1	0	6.00	200.00	35.00	17.15
4	0	+1	–1	7.50	200.00	25.00	19.12
5	+1	0	–1	9.00	150.00	25.00	15.19
6	0	0	0	7.50	150.00	35.00	21.89
7	0	–1	–1	7.50	100.00	25.00	18.26
8	+1	+1	0	9.00	200.00	35.00	19.1
9	+1	0	+1	9.00	150.00	45.00	15.91
10	0	+1	+1	7.50	200.00	45.00	16.39
11	–1	–1	0	6.00	100.00	35.00	18.21
12	–1	0	–1	6.00	150.00	25.00	14.29
13	0	–1	+1	7.50	100.00	45.00	20.02
14	0	0	0	7.50	150.00	35.00	22.76
15	+1	–1	0	9.00	100.00	35.00	16.2

Table 4. AVOVA for quadratic model showing interaction between three variables

Source	Sum of square	Degrees of freedom	Mean square	F value	P value (Prob>F)
Model	99.13	9	11.01	13.09	0.0056
A-pH	0.38	1	0.38	0.45	0.5321
B-Speed	0.11	1	0.11	0.13	0.7346
C-Temperature	0.028	1	0.028	0.033	0.8633
AB	3.92	1	3.92	4.66	0.0833
AC	0.000	1	0.000	0.000	1.0000
BC	5.04	1	5.04	5.99	0.0581
A2	58.52	1	58.52	69.58	0.0004
B2	1.48	1	1.48	1.76	0.2416
C2	37.78	1	37.78	44.92	0.0011
Residual	4.21	5	0.84		
Lack of fit	3.81	3	1.27	6.51	**0.1360**
Pure error	0.39	2	0.20		
Cor total	103.33	14			

Table 5. Statistical information of the model

Source	Response
R square	0.9593
Adjusted R square	0.8860
Coefficient of variation (%)	5.06
Standard deviation	0.92
Adequate precision	9.957

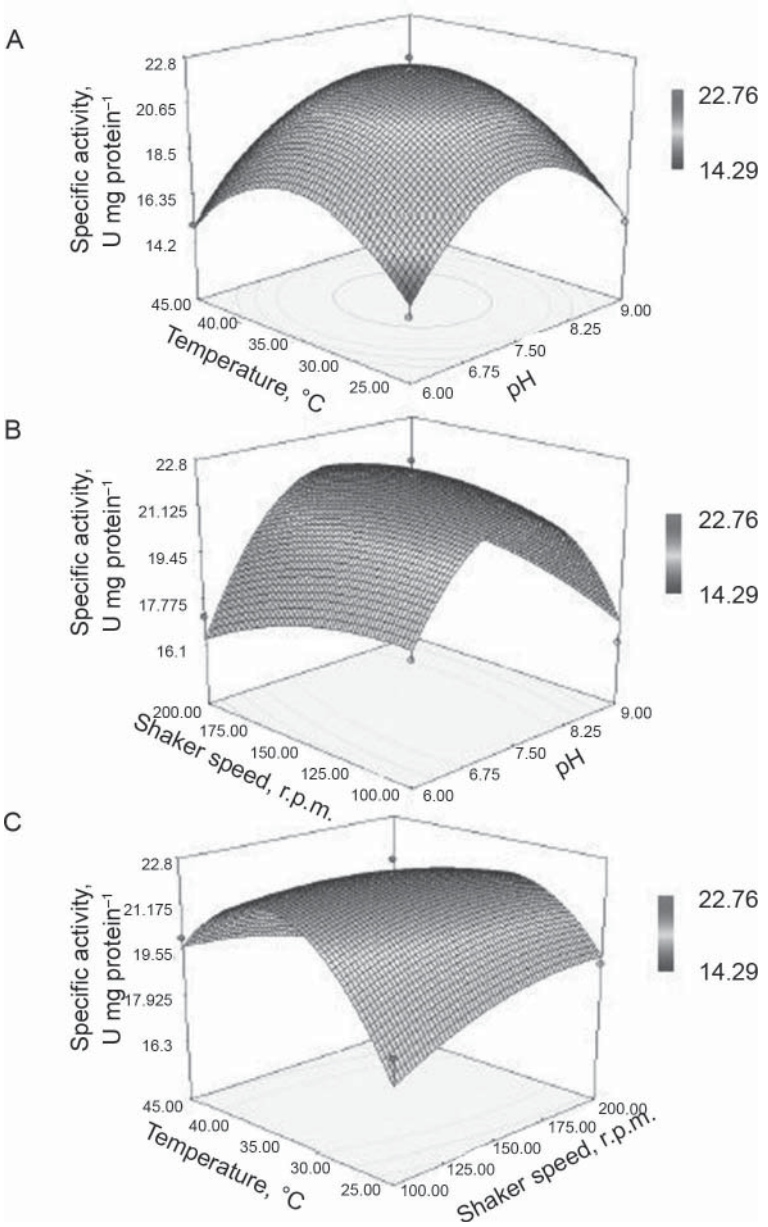

Fig. 3. Response surface graph of β-galactosidase production from the bacterial isolate *Arthrobacter oxydans* SB. 3A: The effect of temperature and pH on β-galactosidase production. 3B: The effect of shaker speed and pH on β-galactosidase production. 3C: The effect of temperature and shaker speed on β-galactosidase production

Optimization of physical factors (temperature, pH, and shaker speed) is one of the most important steps to enhance the production in both laboratory experiments, as well as in fermentation industries. Changes in one of these parameters can have a dramatic effect on the yield of cells and the stability of protein product. The three dimensional surface plots were drawn from the inter-relationship of two variables considering one variable constant. Surface response of the plots indicates the effect of pH, temperature, and shaker speed on β-galactosidase production (Fig. 3). Figure 3A demonstrates the effect of pH and temperature on β-galactosidase production. It was observed that maximum production of β-galactosidase, 20.25 U (mg protein)$^{-1}$, was obtained at pH 6.65 and temperature 39.52 °C. Similarly, Fig. 3B demonstrates the interaction between speed and pH. Here, maximum production of β-galactosidase was 20.53 U (mg protein)$^{-1}$, when speed was 176.7 and pH 6.7. On the other hand, effects of shaker speed and temperature are presented in Fig. 3C, which demonstrates that highest production of β-galactosidase, 21.75 (mg protein)$^{-1}$, was achieved, when shaker speed and temperature were 108.05 r.p.m. and 39.38 °C, respectively. In order to determine the optimum conditions of β-galactosidase production, combined effect of these three variables was also checked. Theoretically the optimum production of β-galactosidase is 21.38 U (mg protein)$^{-1}$ considering pH 6.76, temperature 36.1 °C, and shaker speed 121.37 r.p.m. To validate the data, we have conducted an experiment with these possible values. Results obtained were 22.59 U (mg protein)$^{-1}$ enzyme produced at pH 6.7, temperature 36.0 °C, and shaker speed 122.00 r.p.m. Determination of substrate affinity (K_m) is very important for understanding the kinetic behaviour of enzymes. Our results demonstrated that the K_m of β-galactosidase produced by *A. oxydans* was 10 mM for ONPG as substrate.

Till date, microbial sources of β-galactosidase are preferable in industries due to availability, cost, stability, and high enzyme activity. In our experiment it was detected that β-galactosidase produced by *Arthrobacter oxydans* SB showed highest activity at pH 6.7, temperature 36.0 °C, and shaker speed 122.00 r.p.m. In a similar study Maity and co-workers (2013) stated that the bacterial strain BS1 produced the most β-galactosidase at pH 7.0 and temperature 40 °C; whereas, a few researchers have reported the properties of microbial β-galactosidase at different pH and temperature (Gekas & Lopez-Leiva, 1985; Mahoney, 1985). Dhaked and co-workers (2004) characterized the β-galactosidase production by *Bacillus* sp. and reported the optimum activity at pH 6.8 and temperature 40 °C. Kumar and co-workers (2012) optimized the β-galactosidase production by *Bacillus* sp. through submerged fermentation and reported the highest enzyme activity at pH 7.0, incubation period 48 h, and temperature 30 °C. Thermostable β-galactosidase was also reported by a few researchers that can tolerate temperature up to 90 °C (Volkov et al., 2005; Lauro et al., 2008). So, bacterial β-galactosidase varies greatly in activity, stability at different fermentation conditions, molecular weight, and thus suitable enzymes should be selected according to their application.

3. Conclusions

The use of enzymes in industrial sector significantly increased due to exhaustive industrialization. The present investigation demonstrated the isolation, characterization, identification, and optimization of β-galactosidase production by a bacterial strain *Arthrobacter oxydans*. β-Galactosidase production by this bacterial strain is intracellular, so it is quite difficult to obtain. To overcome this situation, different types of cell wall denaturing

chemicals were applied and Tween 20 was recorded to be most effective. To reduce the industrial cost and time, we have optimized the culture parameters for better production through response surface methodology (RSM) using Minitab 17 software. Due to its high enzyme activity, *A. oxydans* can compete with the other commercially available bacterial strains like *Bacillus subtilis*, *Lactobacillus gasseri*, *Streptococcus thermophilus* and *Anoxybacillus* sp.

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