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Isolation, Characterization and Application of Calcite Producing Bacteria from Urea Rich Soils

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Abstract

Calcium carbonate is one of the most common minerals widespread on earth (4% by weight of the earth's crust). Bacteria are incredibly diverse and abundant and many bacterial species contribute to the precipitation of mineral carbonates in various natural environments. Alkaline pH is the primary means by which microbes promote calcite precipitation which results from the hydrolysis of urea. The study used selective enrichment culture technique to isolate urease-producing bacteria from local urea rich soil and others materials. All isolates were identified using conventional biochemical tests. In addition, all isolates were tested for their ability to enhance the consolidation of sand and compressive strength of mortar as well as absorption reduction properties. One isolate with promising results was selected and optimization of environmental and nutritional conditions was performed. The growth curve of the selected strain with optimized condition was investigated. Thirty three isolates were obtained from the enrichment culture technique. Among them 13 isolates showed increased consolidation of sand. The isolate that showed the highest performance was identified as *Bacillus mycoides*. The optimum pH of the isolate was shown to be 7.0 and an optimum temperature of 35 ^{o}C was found. The growth curve was constructed with a stationary phase starting after 10 hours. The test results indicated that inclusion of *Bacillus mycoides* isolate in cement mortar enhanced the compressive strength, with a maximum increase of 17% in compressive strength and 32% reduction in water absorption was observed with a 28-day mortar sample. In conclusion, locally isolated strain identified as *Bacillus mycoides* enhanced the properties of the cement mortar. It is recommended that a larger scale application of this isolate be implemented.

***Keywords*: Calcite precipitation; Urease, *Bacillus mycoides*; Biocementation; MCP; Palestine**

1. Introduction

Calcium carbonate (CaCO3) is one of the most common minerals widespread on earth, constituting 4% by weight of the earth's crust. It is naturally found in extensive sedimentary rock masses, as limestone, marble and calcareous sandstone in marine, freshwater and terrestrial environment (Ehrlich, 1998; Castanier et al., 1999; Hammes & Verstraete, 2002). Numerous different bacterial species have previously been detected and assumed to be associated with natural carbonate precipitates from diverse environments. The primary role of bacteria in the precipitation process has subsequently been ascribed to their ability to create an alkaline environment (high pH) through various physiological activities (Douglas & Beveridge, 1998; Ehrlich, 1998; Castanier et al., 1999; Castanier et al., 2000; Fujita et al., 2000).

Three main existing groups of organisms that can induce MCP through their metabolic processes are; (i) photosynthetic organisms such as *cyanobacteria* and *algae* that remove CO_2, (ii) sulphate reducing bacteria that are responsible for the dissimilatory reduction of sulphate and (iii) several organisms that are involved in the nitrogen cycle (Castanier *et al.*, 1999; Hammes & Verstraete, 2002; Ariyanti *et al.*, 2012)**.** Urease hydrolyses the substrate urea generating ammonia and carbamate. Carbamate spontaneously decomposes to produce another molecule of ammonia and carbonic acid (Mobley & Hausinger, 1989)**.** The two ammonia molecules and carbonic acid subsequently equilibrate in water with their deprotonated and protonated forms, resulting in an increase in the pH **(Mobley & Hausinger, 1989).** Many organisms can use urea as a source of nitrogen by importing urea into the cell's cytoplasm. One of the most robust ureolytic bacteria is *Sporosarcina pasteurii* (formerly known as *Bacillus pasteurii). S. pasteurii* is an aerobic, spore forming, rod shaped bacterium. It uses urea as an energy source and produce ammonia which increases the pH in the environment and generate carbonate, causing Ca^{2+} and CO_3^{2-} to be precipitated as $CaCO_3$ (Kroll, 1990; Stocks-Fischer *et al.*, 1999; Chahal *et al.*, 2011).

Calcium carbonate precipitation is a rather straightforward chemical process governed mainly by four key factors: (1) the calcium concentration, (2) the concentration of dissolved inorganic carbon (DIC), (3) the pH and (4) the availability of nucleation sites (Hammes & Verstraete, 2002).

The aim of this study is to isolate and characterize strains of urease-producing bacteria that are capable of calcite precipitation and investigate the effect of the selected strain on enhancing the strength of mortar and decreasing permeability.

2. Materials and methods

2.1 Selective enrichment

To screen for strains with high level of urease activity soil, sludge and freshly cut concrete surface samples were collected from different locations in the middle zone of Gaza strip that are likely to contain ureolytic bacteria. To enrich the samples for urease-producing bacteria, 1 g of each sample was inoculated into 50 ml of nutrient broth (250 ml shaking flasks, at 28^{o}C, for 36 hours). The enrichment media consist of 10 g.L^{-1} Yeast extract (YE), 1M urea, 152 mM ammonium sulphate and 100 mM sodium acetate**.** The bacterial isolates were tested for their ability to grow on 5% urea contained on NA. The strains capable of growth at this

concentration of urea were selected and inoculated into higher concentration reaching to 10% of urea concentration. Finally, the strains with the tolerance to the highest urea concentration were selected and used in subsequent experiments. All isolates were introduce to sand column to perform sand consolidation, the cementaion solution was used in consolidation process was 1M of urea and 0.75M of $CaCl_2.2H_2O$. This work was performed the effect of bacterial isolates on different parameters as a following:

2.2 Compressive strength test

All isolates were grown in NB media for 24 hour and suspended in saline buffer, and the isolates which showed higher strength in sand consolidation (designated as TN1B, TN3B, TN5A and TN3E) were suspended in phosphate buffer. Mortars cubes with each buffer contain no cells were prepared and regarded as control 1 and control 2 by using cementation solution and tap water respectively.

The cement to sand to water ratio was 1:3:0.5 (by weight). All components were thoroughly mixed with bacterial inoculums with a buffer by using slandered motor mixer 65-L0005. The mortar cubes was left in the hydraulic shrinkage of cement mortar 65-L0010/B for 24 hour. Compressive strength test of saline buffer was performed interval with 3, 14 and 28 day and phosphate buffer with interval 3, 21 and 28 days.

2.3 Water absorption

To determine the increase in resistance towards water penetration, all mortar specimens (saline and phosphate mortar cubes) were cast and cured in tap water for 28 days, saturated overnight in water and weighed. The bricks were then dried in an oven at 100 ^{o}C for 24 h, cooled and weighed again. Water absorption was calculated by using following formula:

$$\% \text{ Water absorption} = \frac{(W_{\text{saturation}} - W_{\text{Oven dried}})}{W_{\text{Oven dried}}} \times 100$$

Where $W_{saturation}$ is the weight of bricks after saturation in water for 24 h, and W_{Oven} dried is the weight of bricks after oven drying for 24 h (**Sarda *et al*,. 2009)**.

2.4 Optimization of *B. mycoides* growth conditions

2.4.1 Temperature optimization

NB medium was prepared and distributed into several flasks. A 3 ml of an overnight culture was used to inoculate 30 ml media in a 250 conical flask**.** 4 flasks were prepared and incubated at the following temperature (20, 25, 30, 35) and were incubated for 20 hour. Samples were collected after 20 hour of incubation to measure the optical density spectrophotometrically at 660 nm. The experiment was done in triplicate and the average absorbance was recorded.

2.4.2 Optimum pH

NB medium was prepared and distributed into several flasks. A 3 ml of an overnight culture was used to inoculate 30 ml media in a 250 conical flask**.** The pH of the medium was adjusted using 1N NaOH to obtain the following pH values (6, 6.5 7, 7.5, 8, 8.5, 9). An equal volume of the inoculums was added to each flask and incubated at the 35^{o}C for 18 hour.

Sample was collected after 18 hour of incubation to measure the optical density spectrophotometrically at 660nm. The experiment was done in triplicate and the average absorbance was recorded.

2.4.3 Selection of appropriate growth media

Four standard media (Yeast Extract (YE), Beef Extract, Brain Heart infusion Broth (BHB) and Nutrient Broth (NB)) were used to determine the most effective medium for mass cell production of the selected isolate. In addition, two cheap formulation (Rabbit feed and Corn Steep Liquor (CSL) were tested as alternative media for growth. The experiment was done in triplicate and the average absorbance was recorded.

2.5 Bacterial growth curve of *B. mycoides*

An experiment to determine the growth curve of the selected strain *B. mycoides* was carried out using shake flask culture technique to set a growth comparison point at optimized condition. A 3 ml of an overnight culture was used to inoculate 30 ml of Rabbit feed media in a 250 conical flask. The culture was incubated at 35 ^{o}C by shaking for 28 hours at 180 rpm. Inoculation time was considered as zero time. Samples were taken from the culture at different time intervals and used for quantitative determination of growth which was measured spectrophotometrically at 660 nm. Viable cell count was determined as "colony forming units/ml" CFUs simultaneously. A growth curve was constructed by plotting the absorbance at 660 nm against sampling time.

3. Results and discussion

This study was conducted with the aim of isolating locally urease producing bacteria that could be potentially used in various biocementation processes. Urea agar was used for the selection of urease producing microorganisms, producing a red-pink color due to the presence of phenol red, a pH indicator. Based on the qualitative urease productions, 33 isolates were obtained**.** A method to specifically enrich bacteria from most soil within a short cultivation period (36-48 hours), ideally suitable for biocementation. Selection conditions (high pH, presence of urea up to 1 M) have enriched for a superior Bacillus type bacteria that can degrade urea, is highly tolerant to urea and ammonia at high pH and hence ideally suited the biocementation process. From the enrichment cultures, different ureolytic bacterial strains were isolated with high urease activity which is required for biocementation process as was suggested by whiffin, (2004). The isolates were and biochemically identified by using ABIS software (Costin and Ionut, 2007-2013). Although the differences in some bacterial behaviors between the isolates were evident, the most effective isolates were closely related to one another. Similar finding was shown in a previous study Hammes *et al.,* (2003) on identification of ureolytic strains isolated from various environmental locations. This close relationship between the isolates might be due to the dominance presence of Bacillus species as was confirmed by Fleske *et al*., (1998). Stock-Fischer *et al.,* (1999) have stated that Bacillus species are selected by the isolation and cultivation methods. The phenotypic and biochemical properties of the bacteria isolates were resemble those of Bacillus species reported previously (Stocks-Fischer *et al.,* 1999).

B. mycoides (TN1B) was gram positive with opaque creamy appearance on agar plate, non-motile, and catalase positive. B. mycoides was unable to hydrolyze starch, but hydrolyze casein, and lecithin. Citrate was no utilized by B. mycoides. When subjected to salinity and temperature tests, the selected strain was able to survive 0-10% NaCl when incubated for 72 hour.

The sand columns prepared with all bacterial isolates to get the most efficient isolates for sand consolidation, 13 isolates were found to tightly pack through consolidation process while the control sand column collapsed immediately after opening the plastic column. Table 1 show the results of isolates positive or negative to sand consolidation. The predominance of calcite precipitation in upper most surface area of the sand column might be due to higher growth of bacteria in the presence of oxygen which consequently induces active precipitation of $CaCO_3$ around the surface area (Achal et al., 2010b). Similar results were reported in sand by Whiffin et al., (2007); Achal et al (2010a); Harkes et al., (2010) & Dhamia et al., (2012).

Table (1): Isolates were used in sand consolidation

Isolates	Status	Isolates	Status	Isolates	Status
TN1A	**-**	**TN11A**	**+**	**TN7**	**-**
TN1B	**+**	**TN11B**	**-**	**TN8A**	**-**
TN2A	**-**	**TN13A**	**-**	**TN9A**	**-**
TN2B	**+**	**TN14A**	**+**	**TN13B**	**-**
TN3A	**-**	**TN15**	**-**	**TN14B**	**+**
TN3B	**-**	**TN2C**	**+**	**TN13C**	**-**
TN5A	**+**	**TN3C**	**+**	**TN11C**	**-**
TN10A	**+**	**TN3D**	**+**	**TN14C**	**-**
TN10B	**-**	**TN6**	**+**	**TN3E**	**+**
TN5B	**-**	**TN9B**	**-**	**Control1**	**-**
TN8B	**-**	**TN2D**	**-**	**Control 2**	**-**
TN16	**+**	**TN10C**	**-**		

Control 1: sand + cementation solution - **Control 2:** Sand + distilled water

The compressive strength of cube mortars with saline buffer was significantly increased for some mortar cubes that contained microbial cells. Fig 1 show 28-day compressive strength test results with saline buffer. The highest compressive strength was obtained with mortar cubes TN3C (35.6 MPa) and TN3E (35.9 MPa) prepared with cementation solution that were incubated for 28 days as compared to control 2 (32.8 MPa) prepared with tap water. TN3C and TN3E mortar cubes improvement in the compressive strength were 8.5% and 9.4% respectively compared to control 2.

The compressive strength had significantly increased for 2 mortar cubes that contained microbial cells.

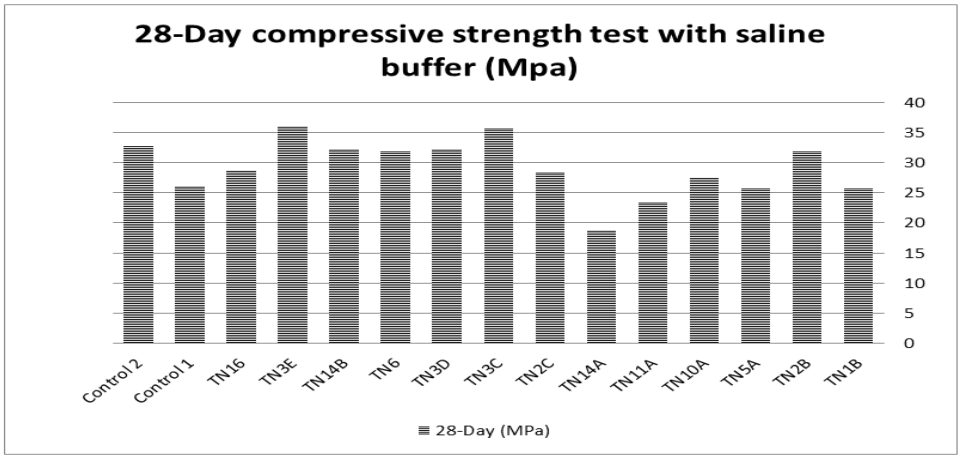

Fig 1 Compressive test results at 28 day for mortar specimens with salinebuffer**.**

Fig 2 show 28-day compressive strength test results with saline buffer. The highest compressive strength was obtained with mortar cubes TN1B (39.23 MPa) and TN5A (38.71 MPa) prepared with cementation solution that were incubated for 28 days as compared to control (33.4 MPa) prepared with tap water. TN1B and TN5A mortar cubes improvement in the compressive strength were 17.3% and 15.89% respectively as compared to control.

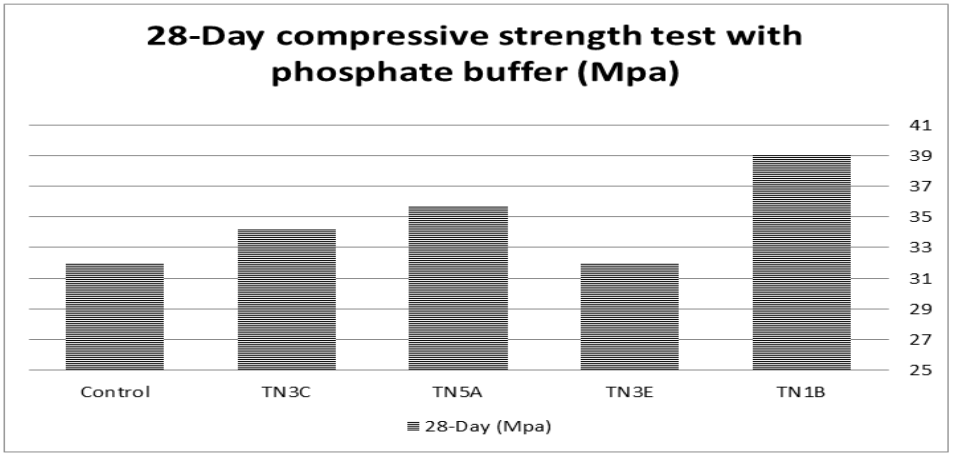

Fig 2 Summarizes the 28 day compressive strength of differentcement mortar specimens with phosphate buffer.

The greatest improvement in compressive strength occurred with B. mycoides isolate in phosphate buffer, there was 17.3% improvement in the compressive strength compared to the control at 28 days. Fig 1 show Portland cement mortar cubes prepared in saline have shown slight decrease in compressive strength in the presence of the cells. The decrease in compressive strength of the cubes containing saline may be due to the presence of chloride ions in the solution, which is known to weaken the integrity of the cement matrix reported previously by Berke et al., (1988). The compressive strength of mortar cubes prepared in the phosphate buffer was consistently higher than the strength of saline-prepared cubes as suggested by Berke et al., (1988).

This improvement in compressive strength is probably due to deposition of $CaCO_3$ on the bacterial cell surfaces and within the pores of cement–sand matrix, which plug the pores

within the mortar as suggested by Ramakrishnan et al., (1998); Ghosh et al., (2005); Achal et al., (2009a); Achal et al., (2009b); Achal et al., (2009c); Achal et al., (2010); Achal et al., (2010a); Achal et al., (2011); Ramachandran et al., (2011) & Vempada et al., (2011).

There was a measurable increase in compressive strength of cement mortar cubes prepared with S. pasteurii, supported by previous studies (Bang & Ramachandran, 2001 & Ramachandran et al., 2001). Thus, it was concluded that the increase in compressive strengths is mainly due to consolidation of the pores inside the cement mortar cubes with microbiologically induced calcium carbonate precipitation.

The influence of bacteria on the water absorption of mortar cubes is given in table 2. The water absorption test was conducted by using saline buffer to determine the increase in resistance towards water penetration in mortars cubes. Mortar cubes treated with bacteria and a calcium source showed significantly less water absorption compared to untreated specimens (control). It can be seen from this table that with the inclusion of all bacterial isolates, water absorption capacity of mortars cubes decreased with compared to control specimens.

Table (2): % Water absorption test with saline buffer

Mortar samples	Weight saturation	Weight dried	% water absorption	% reduction in water absorption
TN1B	129.6	120.1	7.9	19.2
TN2B	133.4	123.1	8.3	14.6
TN5A	131.4	121.4	8.2	15.9
TN10A	116	106.9	8.4	13.5
TN11A	121.3	112.2	8.1	17.2
TN14A	126.3	116.8	8.1	17
TN2C	133.8	123.8	8.0	17.5
TN3C	106.5	98.6	7.9	19
TN3D	123.2	114.1	7.9	18.6
TN6	118	108.5	8.6	11.5
TN14B	124.8	115.3	8.2	15.9
TN3E	138.6	128.5	7.8	19.7
TN16	121.7	112.7	8	18.5
Control	131.1	119.4	9.79	---

The influence of bacteria on the water absorption of mortar cubes with phosphate buffer is given in table 3. The water absorption test was conducted by using phosphate buffer to determine the increase in resistance towards water penetration in mortars cubes. Mortar cubes treated with bacteria and a calcium source showed significantly less water absorption compared to untreated specimens (control). It can be seen from this table that with the inclusion of bacterial isolates, water absorption capacity of mortars cubes decreased with compared to control specimens. Maximum reduction in water absorption test was showed in TN1B isolate**.**

Table (3): % Water absorption test with phosphate buffer

Mortar samples	Saturated weight	Dried weight	% water absorption	% reduction in water absorption
TN1B	126.7	118.5	6.9	32.2
TN3E	119.7	110.7	8.1	20.3
TN5A	125.2	116.7	7.2	28.6
TN3C	128.9	119.3	8	21.1
Control	127.4	115.6	10.2	-

Mortar cubes treated with bacteria and a calcium source showed significantly decrease of the water uptake compared to control specimens. Maximum reduction in water absorption was observed with B. mycodes isolate with phosphate buffer gave 32.21%. Similar observations were made by previous reports by De Muynck et al., (2008) and Achal et al., (2010). Nemati & Voordouw, (2003) & Whiffin, (2004) noticed an additional decrease of the permeability of sandstone cores after injecting $CaCO_3$ forming reactants for a second time. The deposition of a layer of calcium carbonate on the surface and inside pores of the mortar specimens resulted in a decrease of water absorption and permeability. It is clear that the presence of a layer of carbonate crystals on the surface by bacterial cells has the potential to improve the resistance of cementitious materials towards degradation process as suggested by Achal et al., (2010); Achal et al., (2011) & Chahal et al., (2012).

Through previous results we can say that the TN1B isolate which is identified as Bacillus mycoides (B. mycoides) was the best strain capable to increase strength of the cement mortar and has the important role to decrease the water penetration through the mortar compared to the control.

Optimum growth of B. mycoides was at pH 7. Table 4 showed the optimization of pH by using different pH values.

Table (4): Optimizing pH of B. mycoides isolate

pH degree	Spectrophotometer absorbance			
	Trial 1	**Trial 2**	**Trial 3**	**Average**
6	1.431	1.488	1.455	1.458
6.5	1.554	1.512	1.54	1.53
7	1.731	1.698	1.719	1.716
7.5	1.63	1.65	1.67	1.65
8	1.517	1.5	1.48	1.49
8.5	1.375	1.34	1.31	1.34
9	1.29	1.31	1.31	1.308

Table 5 summarizes the bacterial growth levels in different standard and cheap formulation media and showed that the best growth of the bacteria was yeast extract (YE). The cheap formulation "rabbit feed" was superior to nutrient broth which is a well-known favorable media used to grow bacteria at liquid state.

Table 5: Optimization of growth media

Media type	Spectrophotometer absorbance			
	Trial 1	**Trial 2**	**Trial 3**	**Average**
YE	1.827	1.833	1.825	1.828
Beef Extract	1.746	1.721	1.734	1.733
BHB	1.708	1.719	1.727	1.718
Malt Extract	1.362	1.369	1.375	1.368
N.B	1.685	1.679	1.681	1.681
CSL	0.853	0.815	0.832	0.833
Rabbit feed	1.719	1.706	1.825	1.707

Optimum temperature required for growth B. mycoides was 35 ^{o}C, table 6 showed the optimization of temperature by using different temperature degrees.

Table (6): Temperature optimization of the B. mycoides isolate

Temperature	Spectrophotometer absorbance			
	Trial 1	**Trial 2**	**Trial 3**	**Average**
25^{o}C	1.281	1.286	1.276	1.281
30^{o}C	1.509	1.493	1.498	1.5
35^{o}C	1.781	1.815	1.793	1.79
40^{o}C	0.864	0.743	0.724	0.777

Growth curve of the B. mycoides strain was done using the partially optimized conditions Fig 3 shows hour by hour growth curves to B. mycoides isolates which is considered as the selected isolate of this research project. Growth curve for B. mycoides isolate was constructed by plotting the OD_{660nm} on the Y axis and incubation time on the X axis. The maximum OD was seen between 6 to 10 hours and referred as log phase. Spectrophotometer reading showed that the cultures reached the stationary after 10 hour.

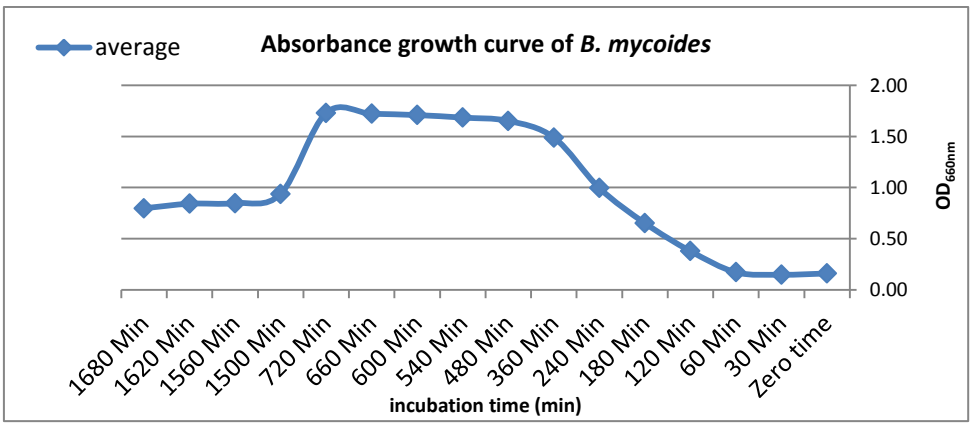

Figure 3: Hour by hour of growth curve of B. mycoides isolates

Conclusions

Thirty three isolates of urease producing bacteria were isolated from sixteen soil samples. Thirteen isolates were shown to increase strength and consolidation of sand samples. Among them B. mycoides isolate showed the highest results in sand consolidation, compressive strength and water absorption test. Rabbit feed was shown to be a good growth medium. It is recommended to further optimize all growth conditions.

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