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Phenol biodegradation by bacterial strain O-CH1 isolated from seashore

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ABSTRACT

Phenol and phenolic compounds are among the most recognized environmental pollutants which exist in industrial wastewater and enter the biological cycles due to the solubility in water. Bioremediation is one of the cost-effective and Eco-friendly methods for phenol removal. In this study, the most effective phenol-degrading bacterial strain was isolated and identified from the shores of the Oman Sea by 16S rDNA. The optimal conditions of various factors, such as pH, temperature, carbon to nitrogen ratio and salinity for the phenol biodegradation, were determined using the experimental design based on Taguchi method with L_9 array (3^4). Ability of the isolated strain (*Halomonas elongata* strain O-CH1) in degradation of different phenol concentrations was analyzed. The optimum operating conditions for phenol removal were determined in pH value of 8, temperature of 35 ˚C, carbon to nitrogen ratio of 100:30 (g/L) and salinity of 35 (g/L). In these conditions, 97% of the phenol was removed from the mediums. According to the optimization results, salinity and pH were the most influential factors in the biodegradation of phenol. The O-CH1 was able to grow and degrade phenol at concentrations of 250 mg/L to 1500 mg/L. Considering the high potential of this strain for phenol degradation, determining the optimal conditions for the biodegradation and its efficacy at high concentrations of phenol, the findings in this study can be used in the biological treatment of phenolic wastewater.

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INTRODUCTION

Nowdays, environmental pollution has become one of the major concerns of societies with the increase industrial growth. Aromatic compounds are environmental pollutants that exist in different regions such as freshwater, sea and land (Deng *et al.,* 2018). Phenol and phenolic derivatives are among the most well-known aromatic compounds that are hazardous due to teratogenic, toxic, carcinogenic and mutagenic properties, and their removal from the environment is important (Rucká *et al.,* 2017; Youssef *et al.,* 2019). Some of the phenol removal methods are optical oxidation, coagulation, adsorption, chemical oxidation, solvent extraction, and biodegradation. Biodegradation is an affordable method with less harmful effects on the environment compared to other methods. In this method, in addition to removing the pollutant, no secondary harmful compounds are produced (Ren *et al.,* 2017). The ability of phenol-degrading microorganisms has been evaluated in various studies. The microorganisms which have been isolated and purified from different regions are as; *Pseudomonas putida* (Kumar *et al.,* 2005), *Candida aquaetextoris* (Jiang *et al.,* 2015), *Pseudomonas aeruginosa* (Hasan *et al.,* 2015), *Thauera phenolivorans* (Yin *et al.,* 2017), Citrobacter sp. (Deng *et al.,* 2018), and Pseudomonas sp. ATR208 (Sepehr *et al.,* 2019). The efficiency of biodegradation depends on several factors, such as nutrients, pH, concentration of the pollutant, and temperature (Margesin *et al.,* 2001; Mohajeri *et al.,* 2010; Hossen *et al.,* 2019). In order to achieve the best performance in biodegradation, the proper organism should be used in optimum conditions. For this purpose, it is necessary to optimize the physical and chemical conditions in which the organism performs the biodegradation (Sadeghi Haddad Zavareh *et al.,* 2016). The traditional methods for determining the optimal conditions, including one-factor at any time, are time consuming and costly. These methods are not capable of examining the simultaneous effects of factors on response, and since the total number of experiments is so high, it is not economically feasible to check them all. Therefore, it is necessary to design the experiments, in which a fraction of the tests is performed using statistical methods. An experimental design based on different models and statistical methods makes it possible to examin different factors simultaneously, in addition to conducting fewer experiments (Samimi and Shahriari Moghadam 2018). Optimization of biodegradation using Taguchi method has been evaluated in many studies such as: optimization of polycyclic aromatic compounds by Sphingomonas sp. (Chen *et al.,* 2008), optimal biodegradation of 4-chlorophenol by *Candida tropicalis* PHB5 (Basak *et al.,* 2013), optimization of crude oil biodegradation by Marinobacter sp. (Shahriari Moghadam *et al.,* 2014), and optimization of biological treatment of chlorpyrifos contaminated coils (Pant and Rai, 2018). Isolation of efficient bacteria, as well as determination of optimal conditions for their performance lead to achieving high efficiency in biodegradation. The main objective of the present study was to isolate and identify most effective phenol-degrading bacterial strain from the shores of the Oman Sea in Chabahar city, Iran. The optimal conditions for various factors involved in phenol biodegradation by isolated bacterial strain were determine using the experimental design based on Taguchi method. Moreover, the ability of the purified strain in degradation of different phenol concentrations was analyzed. This study has been carried out in the Reserch Laboratory of Kermanshah University of Technology and Zabol University, Iran in 2019.

MATERIALS AND METHODS

Isolation of phenol-degrading bacteria and its ability in phenol biodegradation

Aerobic layer sediments along with seawater from different parts of the coast of Chabahar city were randomly sampled in sterile bottles, stored at 4 ˚C and transferred to the laboratory. 5 g of the sediment samples was added to 50 ml of the mineral salt medium (MSM) containing phenol as a carbon source (200 mg/L). The MSM contains 0.5g K_2HPO_4, 1g NH_4Cl, 0.01g $FeSO_4.7H_2O$ as well as 1 ml of micro elements in 1000 ml of sea water. The solution of micro elements contains 70mg $ZnCl_2$, 100 mg $MnCl_2.4H_2O$, 200mg $CoCl_2.6H_2O$, 100mg $NiCl_2.6H_2O$, 20mg $CuCl_2.2H_2O$, 50mg $NaMoO_4.2H_2O$, 26mg $Na_2SeO_3.5H_2O$, 10mg $NaVO_3.H_2O$, 30mg $Na_2WO_4.2H_2O$, 1ml HCl (25%) in 1000 ml distilled water (Schlegel, 1992). After observing the growth in the medium, 5 ml of the medium was added to the new conical flask containing 50 ml of fresh MSM, and the process was repeated 3 times. After serial dilution, 1 ml of the medium was cultured on plates containing nutrient agar medium. Finally, different strains were

purified using the serial dilution method. The ability of isolated bacterial strains to biodegrade phenol was also evaluated. For this purpose, the bacteria were inoculated first into the nutrient broth medium and incubated for 24 hours. After centrifugation, the bacteria were used for inoculation in the flasks containing MSM along with phenol.

Identification of isolated strain

Identification of isolated strain was carried out using 16S rDNA sequencing. First the purified strain was cultured in a nutrient agar medium. Genomic DNA was extracted by DNA extraction kit (Roche, Germany). In order to determine the concentration of DNA and its purity, DNA absorption was measured at 260 and 280 nm. The proliferation of the 16S rDNA was performed using the primers 27F: 5- AGA GTT TGA TCC TGG CTC AG-3 and 1510R: 5-GGT TAC CTT GTT ACG ACT T-3 (Sadeghi Haddad Zavareh *et al.,* 2016) by Primus 25 advanced® thermocycler, and the nucleotide sequence was determined by Takapu-Zist Gene Molecular Biotechnology Co., Iran. After determining the desired sequences, homology searches were performed using the BLAST database (Shahriari Moghadam *et al.,* 2016).

Determination of the optimal conditions for phenol biodegradation

The optimal conditions for the phenol biodegradation by isolated bacterial strain were determined by the experimental design based on Taguchi method with L_9 array (3^4). The optimal conditions for the main operational factors involved in phenol biodegradation such as pH, temperature, carbon to nitrogen ratio and salinity were evaluated. Table 1 shows the range of levels of effective factors, and design of the experiments for the variables based on L_9 array (3^4) is presented in Table 2. Experiments were performed in 250 ml sterile flasks in a shaker at 140 rpm. To reach a number of bacteria required for each treatment, the purified strain was proliferated in a nutrient broth medium. After centrifuging the medium, the saline serum was added, and to reach the final OD of 0.1, the bacteria were inoculated to 50 ml of MSM containing 200 mg/L of phenol. After 4 days, the phenol removal by bacterial strain was measured in different treatments. Non-inoculated culture medium was used for non-biological removal. All the experiments were performed in 3 replicates.

Effect of phenol concentration on bacterial growth and phenol degradation

The O-CH1 was inoculated in a conical flask containing 50 ml of MSM and exposed to different amounts of phenol (250, 500, 750, 1000, 1250, 1500 mg/L) as the only source of carbon and energy. The amounts of phenol removal and bacterial growth (optical density) were measured every 12 hours.

Table 1: The range of levels of various factors

Factors	Symbol	Range of levels		
		1	2	3
pH	A	6	7	8
Temperature (°C)	B	20	25	35
Carbon to nitrogen ratio (g/L)	C	100: 10	100: 20	100: 30
Salinity (g/L)	D	15	25	35

Table 2: Design of experiments for independent variables based on L9 array (34)

Run No.	Manipulated variables			
	A	B	C	D
1	1	1	1	1
2	2	2	2	1
3	3	3	3	1
4	1	3	2	2
5	2	1	3	2
6	3	2	1	2
7	1	2	3	3
8	2	3	1	3
9	3	1	2	3

All experiments were carried out according to the optimal conditions obtained in the previous steps, and the flasks were inocubated in an orbital shaker at 140 rpm.

Measurement of phenol degradation

In this study, phenol concentration was measured by the colorimetric method. Phenolic samples were coupled with 4-amino anti-pyrene, and oxidation in alkaline conditions was carried out using potassium ferricyanide. The red color produced in this reaction was measured at 500 nm. The standard curve was used to measure the amount of phenol remaining in the solution (APHA, 1998).

Statistical Methods

The Statistica 8.0 software was used to design the experiments based on the Taguchi method (Montgomery, 2017). The factorial ANOVA of the design module was used to identify the factors affecting the amount of phenol biodegradation.

RESULTS AND DISCUSSION

Four bacterial strains, capable of phenol biodegradation, were isolated from the sediments and water of the coastal area of Chabahar, Oman Sea. The results showed that the bacterial consortium had a better ability to biodegradate phenol than purified strains, so that almost the whole phenol in the inoculated medium was degraded by the bacterial consortium at the end of the experiment. Various studies have shown that biodegradation by bacterial consortium is more efficient than pure bacterial strain. This could be due to the greater ability of the enzyme as well as the co-metabolism processes of bacterial consortium to produce the pure bacterial strains (Sugiura *et al.,* 1996; Casellas *et al.,* 1998). Identification of the most effective purified strain with the ability to biodegradate phenol, called O-CH1, showed that the strain was 98% similar to *Halomonas elongata*. Halomonas sp. includes some species that have a greate ability to decompose various hydrocarbons. Haddadi and Shavandi (2013) reported that Halomonas sp. strain PH2-2 had the ability to biodegrade phenol to a concentration of 1100 mg/L. Mnif *et al.,* (2009) identified a new strain of Halomonas sp. capable of degradation hexadecane, crude oil, motor oil and diesel fuel at a salt concentration of 10%. Other studies have also shown that *H. campisalis* uses benzoate, salicylic acid, *H. eurihalina* from phenol and crude oil as the only source of carbon and energy (Peytone *et al.,* 2007; Calvo *et al.,* 2002). In addition, phenol biodegradation by *H. organivorans* (Bonfá *et al.,* 2013) and *Halomonas anticariensis*

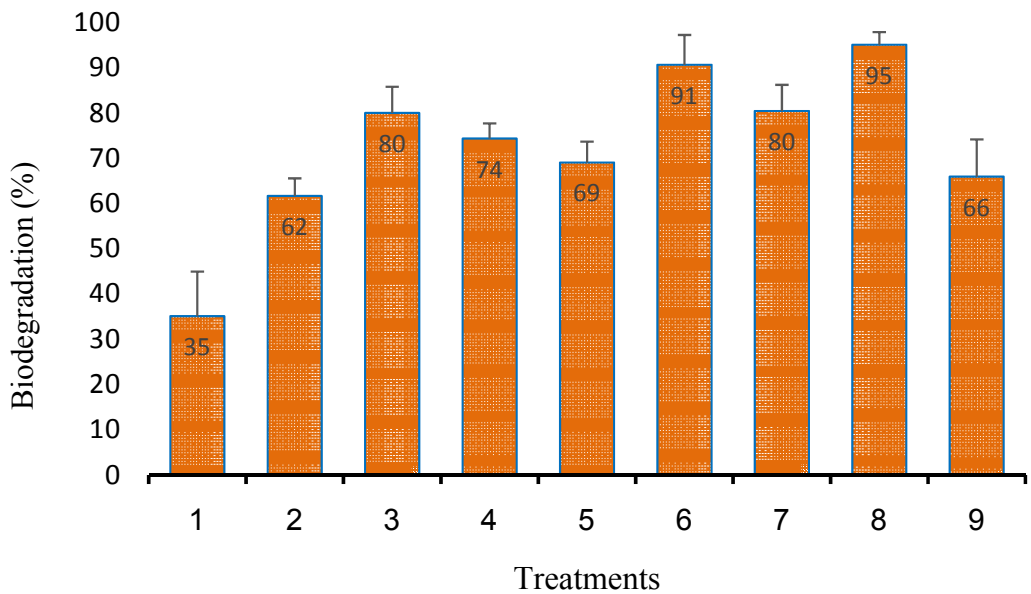

Fig. 1: Phenol degradation percentage by strain O-CH1 in different treatments

FP35 (Tena-Garitaonaindia *et al.,* 2019) has been reported. The results of phenol biodegradation in different treatments showed that the highest and lowest phenol degradation percentages were obtained in treatments No. 8 and No. 1, respectively (Fig. 1).

According to the ANOVA results shown in Table 3, all the studied factors had a significant effect (P <0.05) on phenol biodegradation by strain O-CH1.

The results of different levels of factors affecting the phenol biodegradation by strain O-CH1 are shown in Fig. 2. Among the factors studied based on effect size, salinity had the greatest effect (1.47) and pH (1.12), temperature (0.96) and carbon to nitrogen ratio (0.76) ranked the second, third and forth parameters which were effective in phenol biodegradation respectively.

In different studies, Taguchi method has been used to optimize biodegradation of various compounds such as: optimization of biodegradation of phenol-contaminated wastewater (Hsien *et al.,* 2005), optimization of crude biodegradation by Marinobacter sp. (Shahriari Moghadam *et al.,* 2014), optimization of the factors affecting Malachite Green's biodegradation (Daneshvar *et al.,* 2007), optimization of phenol degradation by *Alcaligenes faecalis* (Kumar *et al.,* 2013) and optimization of phenol biodegradation by *Candida tropicalis* (Basak *et al.,* 2013). In the present study, Taguchi method was used to determine the optimal conditions for the phenol biodegradation by strain O-CH1. The analyses revealed that the optimum operating conditions for the phenol removal were pH value of 8, temperature of 35 ˚C, carbon to nitrogen ratio of 100:30 (g/L) and salinity of 35 (g/L). Under these conditions, 97% of phenol in the medium was degraded by strain O-CH1. The salinity, which has the greatest impact on the amount of phenol biodegradation, depends on environmental conditions such as sediment type, bacterial population, type of compound, etc. Salinity not only affects the metabolism of degrading bacteria,

Table 3: ANOVA analysis for the phenol biodegradation by strain O-CH1

Factors	$p-$value	$F-$value	Sum of squares
A	0.00	27.84	55.91
B	0.00	15.02	30.16
C	0.03	3.96	7.96
D	0.00	35.91	71.82

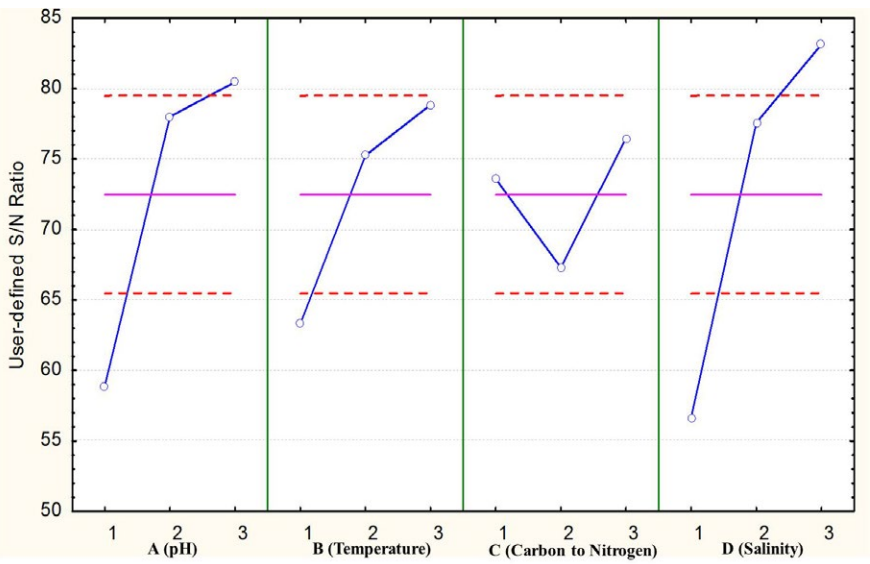

Fig. 2: Effect of different levels of factors on the phenol biodegradation by strain O-CH1

but also affects the solubility of cyclic compounds (Tremblay *et al.,* 2005). Another factor affecting biodegradation is pH of the environment, which was determined to be 8 for optimum conditions for phenol degradation by O-CH1 strain in this study. The pH of the environment leads to changes in bacterial growth, enzymatic activities, intracellular transport, and the degree of solubility of food (Lin *et al.,* 2010). In marine environments, the pH is often constant and partly alkaline. Generally, the optimum pH varies according to the type of microorganism. Some bacteria, such as Mycobacterium sp., perform better at acidic pH due to the greater penetration of cyclic compounds into them, whereas Pseudomonas genus prefers neutral pH for organic matter decomposition (Kim and Freeman, 2005). Other studies have shown that *Rhodococcus pyridinivorans* shows the best biodegradation performance of phenol at pH 8 (Shahriari Moghadam *et al.,* 2016). The results of determining optimum conditions of phenol biodegradation showed that the highest amount of phenol removal was obtained at 35 ˚C. In general, temperature plays an important role in controlling the properties and rate of hydrocarbon metabolism. Temperature changes the population and structure of bacteria, the rate of metabolism, and the physical and chemical properties of pollutants (Atlas, 1981). Previous studies have

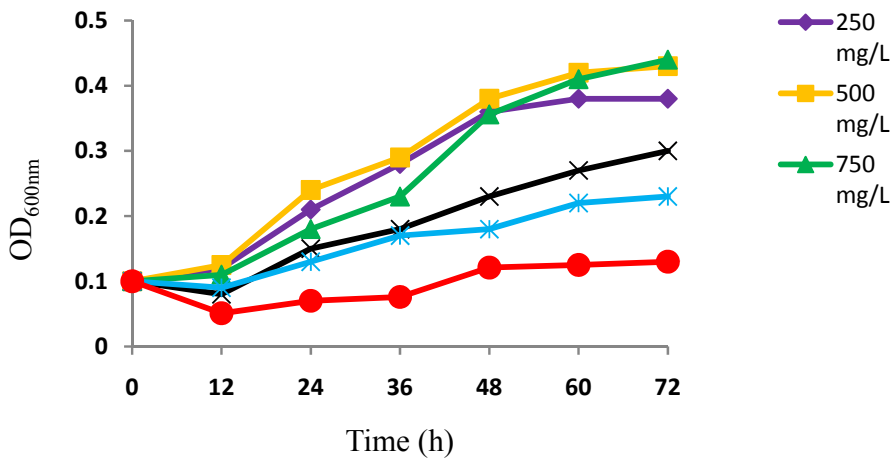

Fig. 3: Bacterial growth of strain O-CH1 in different concentrations of phenol

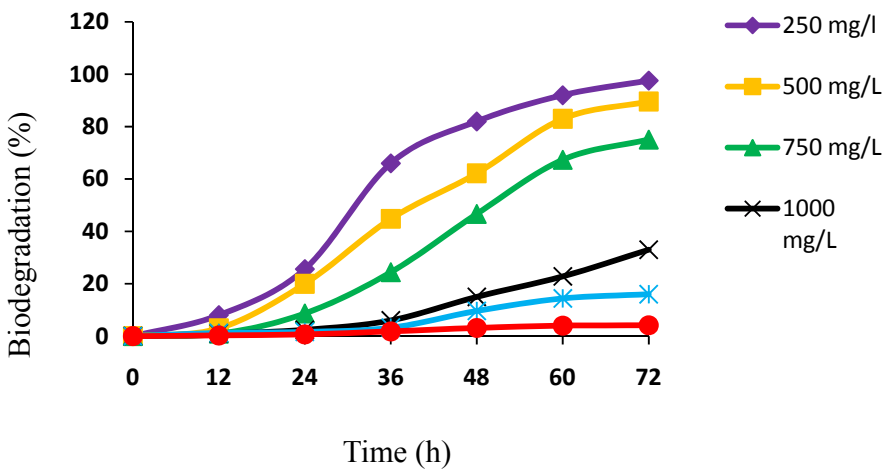

Fig. 4: Percentage of phenol degradation by strain O-CH1 in different concentrations of phenol

shown that most of the mesophilic bacteria have the highest efficiency in decomposing petroleum hydrocarbons in the temperature range of 30 to 40 ˚C (Leahy and Colwell 1990). Similar results were obtained for the biodegradation of phenanthrene in river sediments (Yuan *et al.,* 2001). The results obtained in the present study confirm the results reported in the above-mentioned studies. Nutrients are one of the most important factors in the biodegradation of cyclic compounds, and nutrient deficiencies decrease the number and efficiency of bacteria. In bioremediation, it is important to determine the optimum nutrient concentration to reduce the environmental damage (Bao *et al.,* 2012). In the present study, 100:30 carbon to nitrogen ratio was determined for the efficient decomposition of phenol. Different bacteria at different concentrations of nitrogen source have their own optimal performances and it is important to determine the lowest concentration of nitrogen source where the bacteria perform best. Leys *et al.* (2005) showed that a low ratio of nitrogen to carbon decreases the bacterial growth. The results obtained from determination of optimal conditions of phenol consumption by strain O-CH1 also confirm the above results. In fact, the rate of biodegradation increased with the increase of the nitrogen source. In general, the rate of substrate degradation depends on the substrate concentration and the type of substrate degrading organisms (Okpokwasili and Nweke, 2006). Strain O-CH1 was able to grow and degrade at various concentrations of phenol (Figs. 3 and 4). Growth rate was increased with the increase of phenol concentration up to 750 mg/L, however at higher concentrations, as the lag phase and the substrate inhibitory effect increased, the growth rate was decreased. Other studies have also shown the increase of phenol percentage with the increase of substrate concentration (Shahriari Moghadam *et al.,* 2016), which confirms the results obtained in the present work.

CONCLUSION

In the present study, *Halomonas elongata* O-CH1 as the most effective phenol-degrading bacterial strain, was isolated and purified from the Oman Sea-Chabahar coast. Compared to purified strains, bacterial consortium had a better ability to biodegradate phenol, so that almost the whole phenol in the inoculated medium was degraded by the bacterial consortium. All the experimental factors had a significant effect on the rate of phenol biodegradation by strain O-CH1. Among the factors selected based on the rate of effect, salinity and pH were the most important factors in the biodegradation of phenol. Optimum operating conditions for phenol removal were determined as pH value of 8, temperature of 35 ˚C, carbon to nitrogen ratio of 100:30 (g/L) and salinity of 35 (g/L) based on Taguchi method with L_9 array (3^4). Under optimal conditions, more than 97% of the phenol was degraded. Given the high potency of purified strain in phenol degradation and determination of optimum conditions as well as its contribution to bacterial growth and biodegradation in high amounts of phenol, the results of this study can be used in the bioremediation of the water contaminated with phenolic compounds.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

ABBREVIATIONS

A	pH
ANOVA	Analysis of variance
B	Temperature
C	Carbon to nitrogen ratio
$CoCl_2.6H_2O$	Cobaltous chloride hexahydrate
$CuCl_2.2H_2O$	Cupric chloride dihydrate
D	Salinity
$FeSO_4$	Ferrous sulfate

$Fig.$	Figure
g	Gram
h	Hour
K_2HPO_4	Dipotassium phosphate
l	Liter
mg	Milligram
ml	Millilitre
$MnCl_2.4H_2O$	Manganese (II) chloride tetrahydrate
$NaMoO_4.2H_2O$	Molybdic acid sodium salt dihydrate
$Na_2SeO_3.5H_2O$	Disodium selenite (IV) pentahydrate
$NaVO_3.H_2O$	Sodium metavanadate hydrate
$Na_2WO_4.2H_2O$	Sodium tungstate dihydrate
NH_4Cl	Ammonium chloride
$NiCl_2.6H_2O$	Nickel(II) chloride hexahydrate
nm	Nanometer
No	Number
OD	Optical Density
rpm	Revolutions per minut
$ZnCl_2$	Zinc chloride
$^{\circ}C$	Celsius
%	Percentage

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