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Improvement of Phenol and Heavy Metal Removal by *Streptomyces Flavabus* BA4 Used for Wastewater Treatments

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Abstract

With increasing of modern application of phenolic compound and their use in dettol preparation, discharge of these dangerous materials is stable for long time, too toxic and had a big risk on soil and aquatic microbes. These phenolic wastes cause severe harms to environment, human being and animals health in addition to the whole aquatic life. This study aimed to improve phenol degradation by *Streptomyces flavabus* BA4 previously isolated from wastewater samples. This aerobic bacterium was belonged to filamentous bacteria with high activity to metabolize phenol carbon source, thus it can remove it from contaminated soil or wastewater. In liquid medium, growth was assayed as mg dry weight while residual phenol concentration was assayed using a colorimetric method. In minimal broth medium, *Streptomyces flavabus* BA4 was grown and the effects of different concentrations glucose, temperature, pH value and incubation period on phenol degradation were determined It was clear that addition of 1 g/l peptone enhanced both growth and phenol degradation was detected. Addition of the electron donor, glucose enhanced growth but decreased phenol degradation at low concentrations (0.0-1.2 g/l). Maximum growth and phenol degradation were recorded at 30◦C in medium with pH 6.5-7 after 7 day of growth. Moreover, this isolate showed resistance to some heavy metal determined specially chromium up to 300 mg/l. Increasing heavy metal concentration decreased growth and dehydrogenates activity. The removal rates of phenol and some heavy metals per mg dry weight of the selected bacterium were calculated for wastewater sample and were ranged from 97% for chromium to 6% for copper. In conclusion, *Streptomyces flavabus* BA4 is a promising phenol degraded bacterium and adjusting some physical and biochemical factors enhancing both growth and removal process. The previous *Streptomyces* isolate can be used as a promising treatment for removal of phenol and some heavy metals, especially chromium which is a very toxic metal in wastewater.

Keywords: phenol, Heavy metal, Chromium *Streptomyces*, degradation, wastewater

Introduction

Despite the fact that the aromatic phenolic compounds and their derivatives are either man made or naturally found materials occurring in plants or broadly distributed everywhere in the environment from oil refineries, coking plants, pharmaceuticals, and plastic industries and considered as a priority pollutants causing a severe problems to the sewage network pipelines (ATSDR, 2003, McCall et al., 2009; Park et al., 2012, Salem et al., 2021). Phenolic materials are characteristic pollutants due to their frequent presence in effluents of many industrial processes and are important intermediate products, produced mainly during the degradation of aromatic hydrocarbons, amino acid

or polymers with aromatic rings like lignins and tannins. Phenols is very toxic to living organisms at low concentration and are found mainly in soils, freshwater, sea, wastewater and sediments and phenol pollution is produced from various chemical industries and their wastewaters (APHA, 2005, Basha et al., 2010, Kolhe et al., 2015).

Phenol coefficient is one of the methods to determine the effectiveness of a disinfectant. The harmful effects of phenol on metabolic reaction are complex and generally mostly depend on the temperature used, concentration and the characteristics of the influent, such as pH, as well as presence of other toxic materials and/or suspended solids. At high phenol concentration, humans' disorders in the central nervous system, myocardial depression, irritation of eyes, swelling, corneal whitening, blindness, cardiovascular diseases and gastrointestinal damage were reported (Govindarajalu, 2003, Deng et al., 2018). Mostly, phenol toxicity results from entering into the food cycle causing cell lyses or inactivation of some important enzymes which are mainly protein molecules, responsible for all metabolic reactions. Also, phenols accelerate chemical modifications in a cell wall or alterations in the plasma membrane or nucleic acids, proteins or increased the oxidative stress (Yaoa et al. 2008). The mainly protocols for elimination of phenol from wastewater treatment processes are degradation or removal through chemical reactions (Qodah 2006).

The maximum permissible level for phenol in the environment is 0. 01 mg/l and in tap water is below 1-2 µg/l (Nuhoglu and Yalcin, 2005; Saravanan et al., 2008, Gami et al., 2014). Variety of treatments, such as adsorption, solvent extraction, Wet oxidation, and hydrogen peroxide removal were used for removal process. Fenton's reagent has been employed to eliminate phenol from the polluted samples (Lin and Chuang, 1994), chemical oxidation, and incineration (Wu et al., 2005). But these methods are complex, high cost, and not environment friendly (Yan et al., 2006; Bai et al., 2007; Zhai et al., 2012). Biodegradation is the best way to get rid of phenol since this chemical and physical biological process is cheap, environment-friendly, and easy to handle (Tay et al., 2005; Basha et al., 2010).

Bacteria, fungi, algae and some agricultural wastes recorded as an eco-friendly, effective and low cost material option which effectively used safely. Many aerobic bacteria capable of phenol degradation were isolated, but only few anaerobic bacteria were described. Three novel nitrate reducing bacteria from the genus *Azoarcus* were isolated from three different geographic area in USA and were used phenol as a sole source of carbon (van Schie and Young (1998). Studies showed that phenol is toxic to bacteria and in phenol contaminated sites bacteria can adapt themselves to certain phenol concentrations, but increasing these concentrations, lowered degradation process (Dean-Ross, 1989). *S*ulfate-reducing bacteria, denitrifying bacteria and methanogenic bacteria are described as phenol-degrading isolates which grow in broth medium containing phenol as the only carbon source (Bakker et al., 1977, Boopathy et al., 1995). Two denitrifying bacteria, *Thauera aromatic* and *Thauera selenatis* used phenol as carbon and energy sources (Dean-Ross and Rahimi, 1995). Three new phenol-degrading denitrifying microorganisms were isolated from sediments from different geographic locations. Moreover, many phenol-degrading bacteria were isolated like *Bacillus brevis, B. cereus, Cyanobacterium synechococcus, Pseudomonas putida, Gliomastix indicus, Sphingomonas chlorophenolica* (Nair et al., 2008, Singh et al., 2008, Arutchelvan et al., 2006). Other phenol degrading bacteria have been discussed like *Staphylococcus epidermis, Acinetobacter* sp*, A. calcoaceticus. Burkholderia cepacia, Rhodococcus, Xanthobacter flavus, Pseudomonas* sp. Gulosibacter sp (Rehfuss and Urban, 2005, El-Sayed et al., 2003, Lowry et al., 2009, Banerjee and Ghoshal, 2010, Mohite et al., 2010, Ahmad et al., 2012, Zhai et al., 2012, Mahiuddin et al., 2012;

Ahmad et al., 2014 and Liu et al., 2016). Moreover, different species of *Aspergillus, Pseudomonas, Sporophyticus, Bacillus, Phanerochaete*, etc., are well known as efficient genera for removal of many toxic materials. The response of microorganisms towards toxic materials is very important for reclamation of polluted sites (Congeevaram et al. 2007). Bacteria oxidize phenol into CO_2 and H_2O during metabolic processes (Loh and Chua, 2002) and can utilize phenol as the sole source of carbon and energy for their growth (Geng et al., 2006; Tuah et al., 2009; Nair et al., 2008). The microbial phenol degradation focusing on aerobic degradation using specifuc bacterial strains, different methods for improving the phenol degradation rate, effects of various physicochemical factors on degradation process, and mechanisms of degradation were reported (Bhattacharya et al., 2018). The bacterial stains SP-4 and SP-8 from the pulp and paper mill effluent were capable of tolerating phenol up to a concentration of 1600 and 1800 ppm, respectively (Sachan et al., 2019). These strains were found to be efficient amongst the sixteen strains established by checking their capability of phenol tolerance with respect to the incubation time. These strains can be utilized in real-scale systems as identification of phylogenetically closely related species for phenol degradation is an important aspect. This help in treatment of industrial wastes by bacteria which prove to be more economical to reduce the environmental problems. Degradation by bacteria occurred through some bacterial enzymes and their delivery systems like peroxidase enzyme. Hence, the eco-friendly biodegradation processes are time saving, inexpensive catalyst and no harmful products are formed from degradation of phenol. Four major factors affect the bacterial activity like temperature, pH, surface activity, and the presence of interfering substances. An increase in temperature increased bacterial growth and optimal growth is achieved at pH between 6 to 8; thus, the recommended pH for the tests is 7.5. The surface-active compounds like phenol in low concentrations may increase the disinfectant power and interfering substances such as certain salts may delay disinfectant activity (Ononugbo et al., 2018). Therefore, this study determines studying factors affecting growth and phenol-degradation by the selected bacterium and use this bacterium for bioremediation of phenol contaminated industrial wastewater.

Materials and Methods

Streptomyces flavabus BA4, previously isolated from contaminated wastewater on starch-nitrate agar medium (pH 7.0) at 37◦C by Alaidaroos (2021). The previous isolate was preserved on the same medium on slants at 4◦C until used (Khalel et al., 2020).

Growth in liquid medium containing phenol

Streptomyces flavabus BA4 was grown in the presence phenol (0.5 mg/l) as a sole carbon source and different concentrations of glucose (0.0 -1.4 g/l), in 250 ml Erlenmeyer flasks containing 50 ml of the basal mineral broth medium for 7 days (Alaidaroos, 2021) . Inoculation was carried with 2 ml of pre-culture ($6x10^6$ cfu/ml) and all flasks were incubated at 37°C and 120 rpm. Finally, growth as dry weight (mg/l) and percentage of phenol degradation were determined.

Similarly, the effects of temperature (20-50◦C), initial medium pH (5.5- 8.5) and incubation period (1- 10 days) were determined in the previous medium, inoculated with the tested bacterium and growth and phenol degradation were determined for each treatment.

Determination of bacterial growth and phenol degradation

After bacterial growth, cells were collected after centrifugation at 5,000 rpm for 15 min, washed several times with dist. water, oven dried for 3 days at 60◦C until constant weight, weighted and growth for each treatment was calculated as mg/l. Moreover, phenol degradation for each treat-

ment was measured in the culture filtrate using 4-aminoantipyrine method (APHA 2005, Sachan et al., 2019). The increase in the absorbance was measured using UV–Vis spectrophotometer (Systronics UV–Vis spectrophotometer 118) and phenol concentration was calculated from a standard from phenol. All experiments were made in triplicate and mean was recorded.

Growth of the selected isolate on agar medium with different concentrations of heavy metals

Streptomyces flavabus BA4 was screened for heavy metal resistant activities in starch nitrate agar medium containing different concentration of each tested metal (*Cadium Cd, Chromium Cr, Cobalt Co , Lead pb a*nd Copper) at concentrations ranged from 0.0- 300 mg/l). Stock solutions of each heavy metal solution was sterilized separately for 15 min at 110°C (Saurav and Kannabiran, 2009) and agar plates with the selected concentration of the tested metal ion were prepared (Latha et al., 2015). After 10 days of growth of the selected bacterial isolates at 37°C, the mean colony diameter was measured. Growth was reported as high, moderate or poor (Koushalshahi et al., 2012, Daboor et al., 2014).

Growth in liquid medium with different concentrations of Chromium

The tested isolate BA4 was cultivated in 500 ml flasks containing 100 ml of starch nitrite broth medium with different concentrations of Chromium (0.0- 300 mg/l) and after shaking in an orbital rotary shaker at 120 rpm for 10 days, growth, dehydrogenase activity and percentage of Chromium removal were recorded for each used concentration (El- Bestawy et al., 2013).

Quantification of heavy metal

The solutions or wastewater sample were analyzed for metals concentrations or Cr (VI) ions using Plasma Atomic Emission Spectrometer (ICPE-9000) at Center of Excellence in Environmental Studies, KAU, SA. Percentage of heavy metal removal were recorded for each used concentration. It was from the following equation**:**

Percentage of heavy metal removal = Q1-Q2/Q1 x100

Q1: The quantity of heavy metal at the beginning,

Q2: The quantity of heavy metal at the end.

Preparation of immobilized bacterial cells

Immobilized bacterial cells of the pure culture of *Streptomyces flavabus* BA4 was prepared on alginate matrix (Williams and Munnecke, 1981). After growing in starch nitrate medium for 7 days, cells were collected, dried (0.1 g) and mixed with 100 ml of 5% aqueous solution of calcium alginate. The solution was dripped through a capillary tube (3.0 mm diameter) into a solution of 0.2 % $CaCl_2$ (Popa and Asach (2012).

Dehydrogenase activity

The technique described by Weddle and Jenkins (1971) was used to determine Dehydrogenase activity. In a tube containing 10 ml of cell suspension, I ml of milliliter of Triphenyl tetrazolium chloride and few drops of 5 % sodium sulfate solutions were added. After shaking at 30◦C for 30 min. in the dark and centrifugation for 10 min., the absorbance was at 480 nm. (Shim et al. 2003).840

Treatment of the wastewater

Wastewater sample (2000 ml) was collected from Bani Malek treatment plant. Phenol and metal concentrations were recorded as described above. In 1000 ml flask containing 200 sterile wastewater, 10 ml of the most efficient bacterial suspension, prepared after growing in starch nitrate medium for 7 days at 30◦C and 200 rpm and centrifugation to remove the supernatant which was

replaced by wastewater. Three sets of were used to compare the removal efficiencies of the tested bacterium (Jefferson et al. 2001; Gikas 2007, El-Bestawy et al. 2013)

Statistical analysis

Triplicate measurements were carried out in all the cases and mean value was reported ±SD. Any significant difference between sample and control was determined by using t-test at $P < 0.05$.

Results

Streptomyces flavabus BA4 was previously isolated from wastewater sample, obtained from the industrial contaminated area in Jeddah, Saudi Arabia on Starch Nitrate Agar. In liquid medium, the previous isolate grew well on MSA medium with 0.1% phenol as carbon source. Increasing glucose concentration in the medium, increase the growth (dry weight per liter) and decrease phenol degradation (Figure 1). It was noticed that the isolates *Streptomyces flavabus* BAh4 has a dark brown color when grown in liquid broth medium containing phenol. Also, the tested isolate grew well for 10 days and maximum phenol degradation was recorded at 30◦C (Figure 2). Similarly, maximum growth and phenol degradation were noticed in medium with initial pH value ranged from 6.5- 7.5. Increasing medium pH more than 7.5 or decreasing it less than 6.5 decreases both growth and phenol removal (Figure 3). Both growth and phenol degradation increased with time up to 7 days, then become stable and increasing time was not associated with any increase in either growth or phenol degradation (Figure 4). Moreover, *Figure 5 showed phenol* degradation by the actinomycete isolate *Streptomyces flavabus* BA4 grown in *broth medium as free or immobilized o*n Ca alginate. It was noticed that immobilization process enhanced phenol degradation and maximum phenol degradation was reported after 5 days of growth while maximum phenol degradation was reported after 7 days of growth in medium inoculated with free cells (Figure 5).

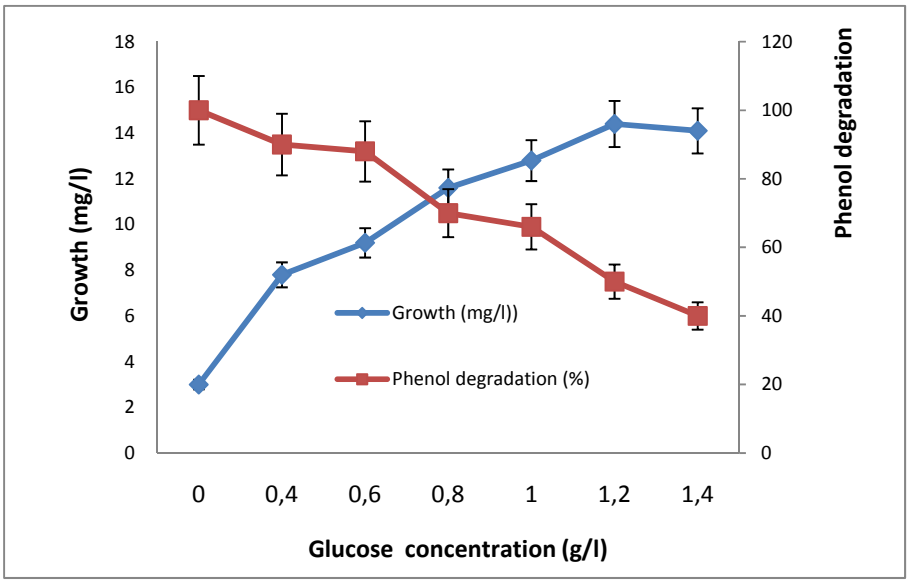

Figure 1. Effect of different concentrations of glucose on growth and phenol degradation by the selected isolate, *Streptomyces flavabus* BA4

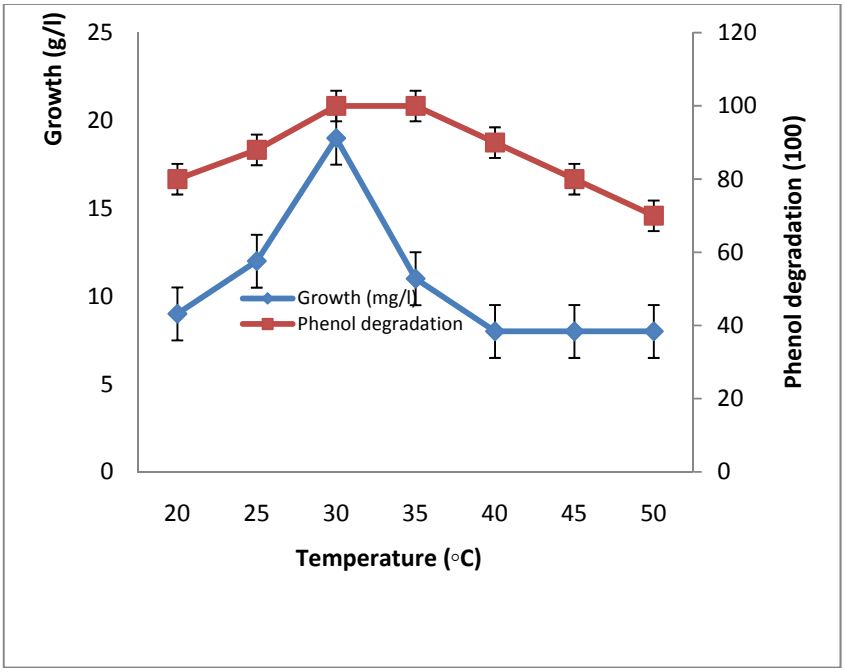

Figure 2. Growth and Phenol degradation by the Actinomycete isolate *Streptomyces flavabus* BA4 grown at different temperature

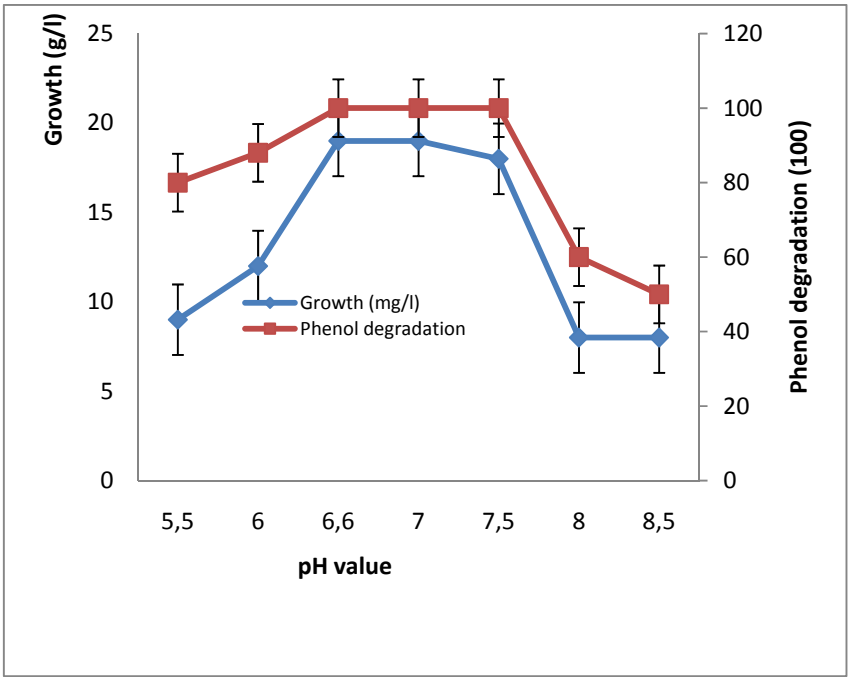

Figure 3. Growth and Phenol degradation by the Actinomycete isolate *Streptomyces flavabus* BA4 grown *i*n medium with different pH values

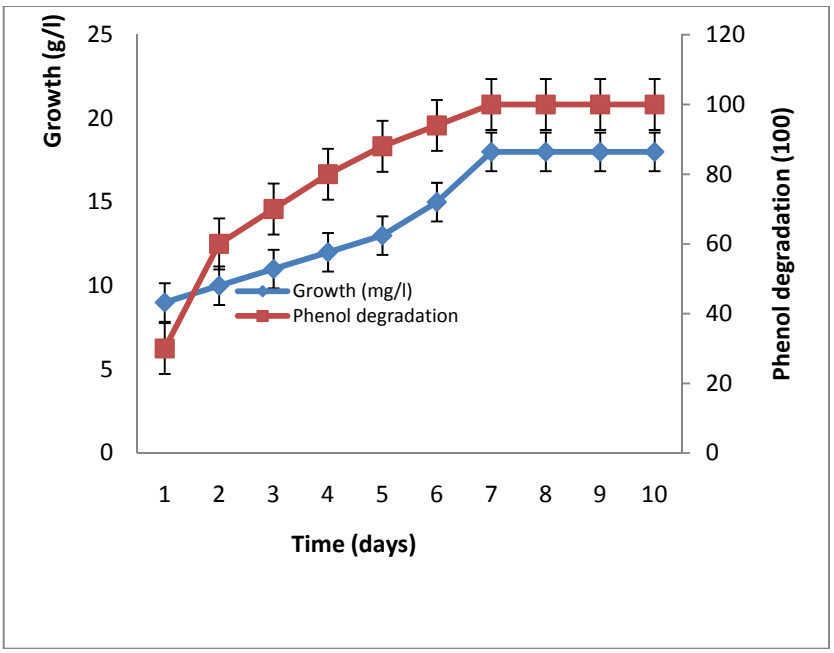

Figure 4. Growth and Phenol degradation by the Actinomycete isolate *Streptomyces flavabus* BA4 grown for different *i*ncubation period.

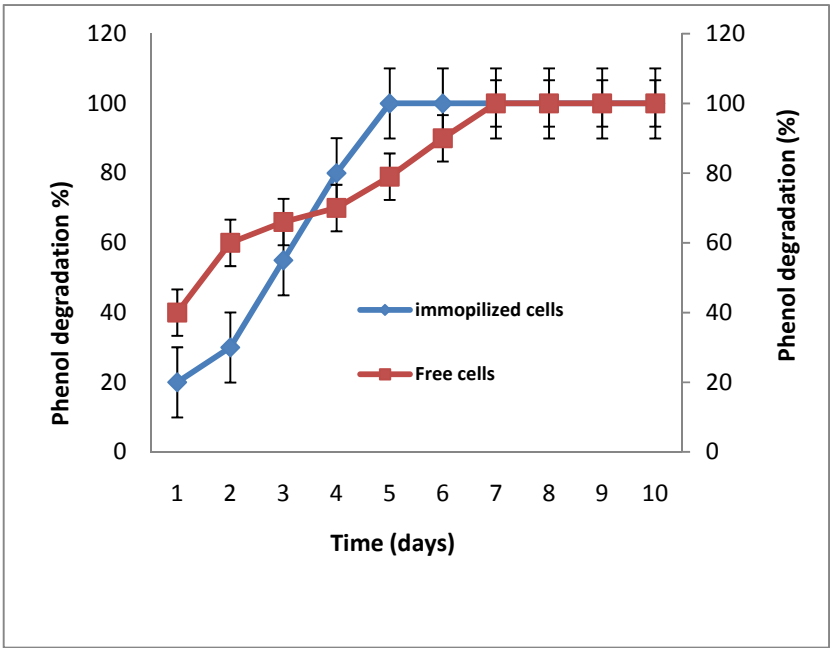

Figure 5. Phenol degradation by the Actinomycete isolate *Streptomyces flavabus* BA4 grown free or immobilized *o*n Ca alginate.

Table 1 showed the growth of the bacterial isolate BA4 on starch nitrate medium with different concentration of heavy metals (50, 100, 150, 200, 250 and 300 mg/l) and compared o control

(without heavy metals). The tested isolate grow with different degrees, heavy growth (+++), moderate growth (++) or poor (+) . Increasing heavy metal concentrations decreased growth and the lowest growth was recorded at 250 mg/l for all tested heavy metals except Cr where the lowest growth was recorded at 300 mg/l.

From the obtained results, the selected *Streptomyces* isolate considered as a tolerant isolate to some heavy metals especially to chromium which at high concentrations, decreased growth, dehydrogenase activity and % of Cr^{++} *removal (Figures 6 a*nd 7). *Thus, this isolate was used to purify a wastewater sample collected* (Table 2). *Removal of* percentages of phenol and heavy metals removal from wastewater sample collected from Jeddah by the isolate *Streptomyces flavabus* BA4 were recorded. Concentrations of phenol and some heavy metals were recorded before and after bacterial treatment and % of removal/mg biomass were calculated. It was clear that phenol, Co and Cr were significantly decreased compared to control with 87%, 54% and 97%, respectively while % of removal/mg biomass were 11.2 %, 10.1% and 16.3, respectively (Table 2).

Table 1. Growth of the selected isolate *Streptomyces flavabus* BA4 on medium containing different concentrations of heavy metals (*Cd^{++}*, *Cr^{+++}*, *Co^{++}*, *Pb^{++}* and Cu^{++}).

	Cd^{++}	*Cr^{++}*	*Co^{++}*	*Pb^{++}*	*Cu^{++}*
50	+++	+++	+++	+++	+++
100	+++	+++	+++	+++	+++
150	++	+++	++	++	++
200	+	+++	+	+ +	+ +
250	-	+	+	+	+
300	-	+	-	-	-

+++: heavy growth, ++: moderate growth, +: poor growth, -: no growth

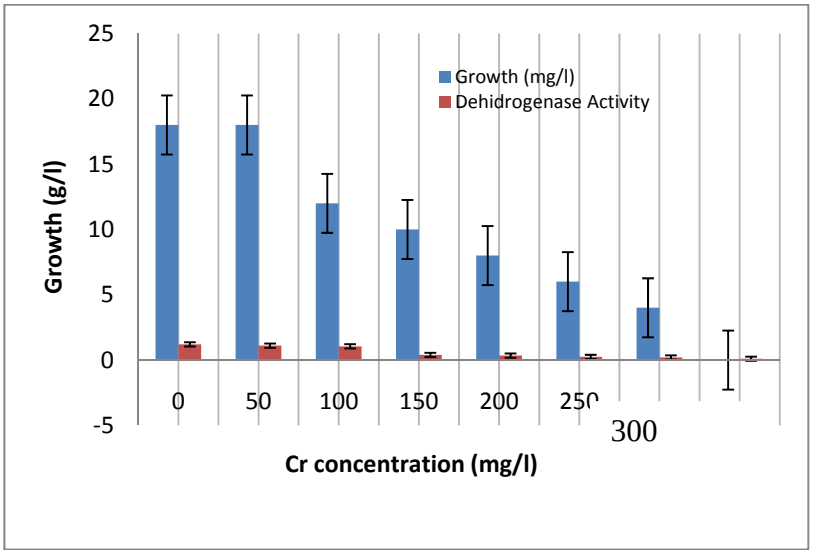

Figure 6. Growth and dehydrogenase activity of the Actinomycete isolate BA4 grown in different concentrations of chromium.

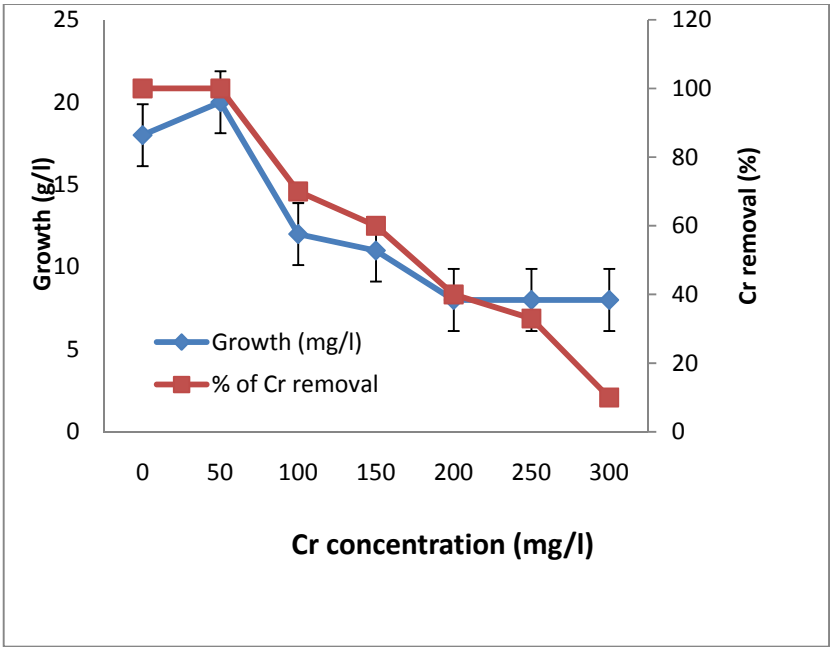

Figure 7. Growth and chromium removal by the Actinomycete isolate BA4 grown in different concentrations of chromium

Table 2. Removal of percentages of phenol and heavy metals removal from wastewater sample collected from Jeddah

	Before treatment (mg/l, control)	After treatment (mg/l)	% of removal	% of removal/mg biomass
Phenol	89±12	11±0.2*	87	11.2
Co^{++}	107±6.1	49±1.1*	54	10.1
Ni^{++}	54±1.8	44±3.0	18	09.1
Cr^{+++}	39±3.1	0.9±3.1*	97	16.3
Pb^{++}	111±9.3	97 ±5.3	30	08.9
Cd^{++}	48±4.6	40±4.6	16	05.9
Cu^{++}	66±3.1	62±1.1	6	02.9

*: significant results at $p \leq 0.05$

Discussion

Nowadays, organic materials like phenol and heavy metal pollution reported as the most important environmental problems particularly in relative to water pollutions. Numerous industrial wastes containing and waste waters are discharged in seas or rivers and damage ecosystems and finally human health. Bio-remediation methods had lower cost and greater efficiency in treating wastewater and are reported as the superior methods compared to physical or chemical methods like ion exchange, heat and chemical treatments, precipitation, and evaporation due to low metal concen-

trations in some cases (Zouboulis et al., 2004, Salem et al., 2021). Due to high surface area to volume ratio, different bacterial genera have excellent roles to remove phenol and metals from wastewater (Ahluwalia and Goyal, 2007). The large contact bacterial surface area provide strong interaction between pollutants and active groups on the bacterial surfaces or bacterial enzymes, finally, these materials are successfully biosorbents or degraded to less toxic or non toxic compounds with small particle size (Zouboulis et al., 2004 and Ziagova et al., 2007). Bacterial cells are able to degrade different compounds in wastewater due to several advantages like stability and excellent activity, high yield and reuse of the bacterial cells (Reischwitz et al., 1995). In this study, *Streptomyces flavabus* BA4 from the actinobacteria was used for the bio-remediation of phenol and heavy metals from waste water sample. Degradation efficiency of phenol compounds by *Streptomyces halstedii* scabies, *S. avendulas* SA2-14, and *S. badius* ATCC 39117 were studied by kim (2000). He reported that *S. lavendulas* had superior ability in degradation of phenol compounds. Also, *Streptomyces lavendulas* was the best strain in degradation ability for lignin and various production of important enzyme. Therefore, he suggested selection of lignin degraded Actinomycetes for removal of phenol compounds. Alaidaroos (2021) obtained eight bacterial isolates which grow in medium supplemented with 0.1% phenol and *Streptomyces flavabus* BA4 was the best in phenol degradation. She added that addition of 1 g/l peptone or vitamin B complex enhanced phenol degradation and the previous isolate BA4 is a promising strain for phenol removal from wastewater. Growth conditions optimization for the isolate *S. flavabus* BA4 enhanced both growth and phenol degradation. On contrast to peptone, addition of glucose decreased phenol degradation which may due to the use of glucose as carbon source instead of phenol. Moreover, phenol degradation process was a maximum at 30◦C and initial pH value ranged from 6.5-7.5. Ononugbo et al (2018) reported that phenol activity was higher on both Escherichia *coli* and *Staphylococcus aureus* at 45◦C than at lower temperature and reduced as the temperature was optimum. At very high and low pH values, the toxic effect of phenol were increased.

Using immobilized cells on Calcium alginate (Ca-alginate), biodegradation process was always enhanced. Ca-alginate is a good supports than synthetic polymers. It was clear that enzymes or whole cells immobilization is better for growth, take less time, act under mild conditions and inexpensive (Aksu and Gonen , 2004, Zhai et al., 2012, Zhang et al., 2021). They added that immortalized cells may be used in a wide range of applications in researches on cellular metabolic regulation due to the cellular characteristics stability and uniformity.

Similarly, microorganisms were used to remove heavy metal contamination whereas *Bacillus* biomass has been used for removal of heavy metal from aqueous solution and decrease the environmental problems connected with textile wastewater (Xu et al., 2011). Heavy metals may be adsorbed on the bacterial surface due to complex formation between the metals and carboxyl, hydroxyl and phenolic surface functional groups of the extracellular polymeric substances (EPS) of different bacterial species (Yuncu et al. 2006). Presence of heavy metals in the growth medium, decrease the growth and dehydrogenase activity (El Bestawy et al., 2013, Sayqa and Ahmed, 2021). Ten marine actinomycetes isolates collected from El-Gona Wastewater Treatment Station, identified as *Kocuria palustris, S. parvus, S. griseorubens, S. rochei, S. albidoflavus, S. griseus* and *Streptomyces* sp. recorded significant activities to treat wastewater. Both *S. griseorubens* and *S. griseus* have excellent roles in efficient treatments of ammonia and phosphates from the used wastewater samples compared to the other isolates (Madkour et al., 2019).

Conclusions

Wastewater treatment in developing countries is an extreme problem and treatment of these wastes is very essential to attain sustainable environmental growth. Actinomycetes group which is filamentous bacteria can be used to treat and purify wastewater and make it less harmful to the environment. In this study, the efficiency of the actinomycete, S*treptomyces flavabus* BA4, in removing of phenol and some heavy metals from wastewaters is studied and the selected isolate showed a promising activity in phenol and heavy metal removal. Thus, the previous bacterial isolates can be used safely to treat wastewater.

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Conflict of Interests: The author declares no conflict of interest exist.

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