THE *BRADYRHIZOBIUM JAPONICUM 273* STRAIN'S ABILITY TO DEGRADE PHENOL. PART 1

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Abstract

Phenol and its derivatives are hazardous pollutants being highly toxic even at low concentrations. Biotechnology has been very effective in dealing with major environmental challenges through utilizing different types of bacteria and biocatalysts to develop innovative processes for the biodegradation, biotreatment and biosorption of various contaminants. Many aerobic phenol-degrading microorganisms have been isolated and the pathways for the aerobic degradation of phenol are now firmly established. In many studies the results showed that Bradyrhizobium japonicum produces the most stable enzymes for biodegradation of organic pollutants. In this study we investigate biodegradation of phenol by Bradyrhizobium japonicum 273 cells. We made experiments with poor and rich (with two organic carbon sources) mineral medium. We compare an aerobic biodegradation in shaking flasks of 250 ml in a rotary shaker with various initial concentration of substrate: 0,02; 0,04; 0,06 and 0,08 g dm^{-3} phenol. The phenol degradation time was 10 days. We investigated the impact of glucose as an additional carbon source on the phenol biodegradation process. The maximum amount of degraded phenol was 1.08 g. It was obtained in an experiment with 0.02 g dm^{-3} initial phenol concentration when working in poor medium. When using a poor nutrient medium, the amount of degraded phenol is bigger than that in the rich nutrient medium. In experiments with a rich medium, Bradyrhizobium japonicum 273 cells grow and, thanks to the second carbon source, glucose, which causes a smaller amount of phenol to be treated.

Keywords*: biodegradation, phenol, Bradyrhizobium japonicum 273 strain, nutrient media*

1. INTRODUCTION

Chemical and petroleum industries generate a wide variety of highly toxic organic pollutants, which have led to cumulative hazardous effects on the environment. The effluents of these industries often contain aromatic organic compounds that are rather resistant to natural degradation and therefore persist in the environment. This makes them capable of long-range transportation and bioaccumulation in human and animal tissues. Phenol is a pollutant that is usually found in many industrial effluents such as wastewaters from coal processing plants, oil refineries, pulp and paper manufacturing plants, resins and coke manufacturing, steel industries, pharmaceutical industries, plastic and varnish industries, textile units, pesticide plants, tannery, and smelting and related metallurgical operations [1, 2]. Contamination of soil, surface water, and underground water by aromatic organic pollutants such as phenol and its derivatives has caused great concern worldwide. Phenols are well known for their high toxicity for human life, aquatic life, and others [3, 4]. They are considered to be among the most hazardous contaminants, and they are certainly the most difficult to remove [5]. Phenol may be fatal by ingestion, inhalation, or skin absorption, as it quickly penetrates the skin and may cause severe irritation to the eyes, the mucous membranes, and the respiratory tract [5]. Oral exposure to phenol may cause severe damage to the liver and kidney and ingestion of 1 g of phenol is reported to be lethal to humans [6].

Phenol and its derivatives are hazardous pollutants that are highly toxic even at low concentrations. The management of wastewater containing high concentrations of phenols represents major economical and environmental challenges to most industries. Biotechnology has been very effective in dealing with major environmental challenges through utilizing different types of bacteria and biocatalysts to develop innovative processes for the biodegradation, biotreatment and biosorption of various contaminants and wide range of hazardous materials. Biological treatment has proved to be the

most promising and most economical approach for the removal of many organic water pollutants such as phenol. Numerous studies have been published in the literature dealing with the biodegradation of phenols utilizing different types of biomasses and different types of reactors [7].

It has been reported that phenol degradation is sensitive to many factors such as temperature, pH, incubation periods, carbon and nitrogen sources, aeration rate, temperature, pH, etc. The pH and temperature required for optimal phenol degradation was 6.5 and 37 ^{0}C respectively [8]. Finding an efficient method to remove phenol effectively has attracted increasing concern for the environmental remediation and the health welfare of human beings [9]. Physico-chemical methods, such as ultra-violet irradiation, ozonation, hydrogen peroxide, Fenton's reagent, or a combination of these methods, have been employed to eliminate phenol in industrial wastewater [10, 11]. These methods were effective in removing phenol but were complex and costly. Hence, the development of improved technologies capable of degrading phenol in the environments is necessary. Alternatively, the biodegradation of phenols is an environmentally friendly and cost-effective technology currently preferred to reduce phenol compounds [12].

Microorganisms capable of degrading phenol are common and include both aerobes and anaerobes. Many aerobic phenol-degrading microorganisms have been isolated and the pathways for the aerobic degradation of phenol are now firmly established. The first steps in the ortho-pathway include oxygenation of phenol by phenol hydroxylase enzymes to form catechol, followed by ring cleavage adjacent to or in between the two hydroxyl groups of catechol. Catechol is converted to cis,cis-Muconic acid by the catechol 1,2-dioxygenase enzyme. Then follow catechol 2,3-dioxygenase enzyme to TCA (tricarboxylic acid cycle).

Phenol can also be degraded in the absence of oxygen. The knowledge of this process is less advanced than that of the aerobic process, and only a few anaerobic phenol-degrading bacteria have been isolated to date. Convincing evidence from both pure culture studies with the denitrifying organism *Thauera aromatic* K172 and with two *Clostridium* species, as well as from mixed culture studies [13].

The ortho and para-hydroxylation pathways of phenol degradation are shown in Figure 1.

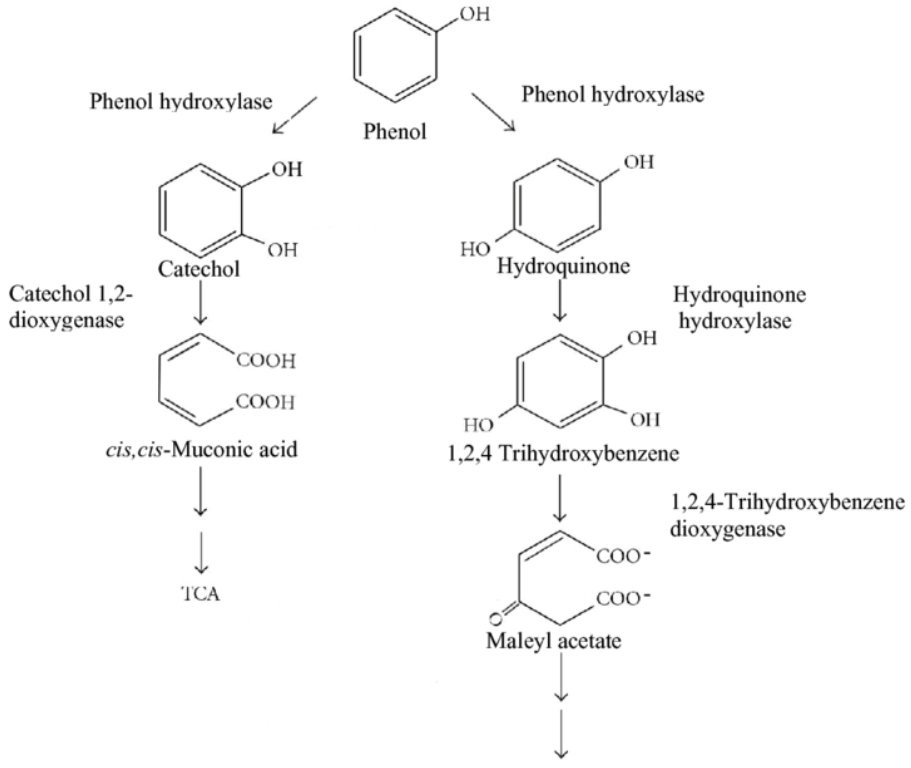


Fig. 1. Ortho and para-hydroxylation pathways of phenol degradation [14].

Based on the theory that a few microorganisms can utilize phenol as the sole source of carbon and energy [15, 16], numerous bacterial species have been isolated and characterized as phenol-degrading microorganisms [17,18]. These bacterial species include *Pseudomonas putida* [19], *Rhodococcus erythropolis* [20], *Bacillus subtilis* [21], *Serratia marcescens* [22], *Arthrobacter citreus* [23], *Alcaligenes faecalis* [24], *Acinetobacter* [25-28]. *Penicillium chrysogenum* [29], *Aspergillus awamori* [30], *Candida maltosa* [31], *Trichosporon cutaneum* [32]. Native microbial species have been reported to be more adaptive and capable of out-competing non-indigenous microorganisms in the remediation of special polluted environments [33].

In many studies the results showed that *B. japonicum* produces the most potent dehalogenase and the most stable enzyme. In our previous research we investigated biodegradation of 1,2-dibromoethane with *Bradyrhizobium japonicum 273* cells [34]. We compare an aerobic biodegradation in shaking flasks with various initial concentrations of substrate of 1,2-dibromoethane (DBE). We analyzed also biodegradation with free and immobilized cells onto a granular polymeric support. Different processes of dehalogenation were carried out in a laboratory bioreactor in presence and absence of constant electric field and influence of electric field was investigated.

The main goal of this study is to explore biodegradation of phenol with *Bradyrhizobium japonicum 273* cells. We made experiments with poor and rich (with two carbon sources) mineral medium. We compare an aerobic biodegradation in shaking flasks of 250 ml in a rotary shaker with various initial concentration of substrate: 0,02 g dm^{-3}; 0,04 g dm^{-3}; 0,06 g $dm^{-3}$and 0,08 g dm^{-3}phenol. The phenol degradation time was 10 days. We investigated the impact of glucose as an additional carbon source on the phenol biodegradation process.

2. MATERIALS AND METHODS

2.1. Bacterial strain

Bradyrhizobium japonicum 273 was obtained from the National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria (NBIMCC). The strain was inoculated in mineral medium (MMY) containing (per liter): 1 g of yeast extract, 0,2 g of NaCl, 0,2 g of $MgSO_4$, 0,5 g of K_2HPO_4, 10 g of Glucose, 1 g of tap water. The medium pH was adjusted to 7.2 before being autoclaved. The inoculum develops up to 24 h at 30 ^{0}C and 100 rpm in a rotary shaker.

2.2. Mineral medium for the fermentation experiments

2.2.1. Poor medium

The poor medium (MMY) contained (per liter): 5.37g of Na_2HPO_4 .$12H_2O$, 1.36 g of KH_2PO_4, 0.5g of $(NH_4)_2SO_4$ and 0.2 g of $MgSO_4.7H_2O$, supplemented with 5 ml of trace element solution per liter: 0,53 g of $CaCl_2$, 0,2 g of $FeSO_4.7H_2O$, 0,01 g of $ZnSO_4.7H_2O$, 0,01 g of H_3BO_3, 0,01 g of $CoCl_2.6H_2O$, 0,0046 g of $MnSO_4.5H_2O$, 0,003 g of $Na_2MoO_4.2H_2O$, 0,002 g of $NiCl_2.6H_2O$. The medium contained only one carbon source – 5 % phenol from 0,02 to 0,08 g dm^{-3} initial concentration for different processes..

2.2.2. Rich medium

Rich medium contained the same mineral like poor medium. For our fermentation experiments we added the second carbon source – 1 g dm^{-3} glucose monohydrate. The vitamin solution was substituted by addition of 0,25 g dm^{-3} Yeast extract. This rich media already contained two carbon sources: 5 % phenol and glucose monohydrate.

2.3. Experimental conditions

Fermentation experiments have been carried out in flasks of 250 ml in a rotary shaker at 30 ^{0}C and agitation speed of 100 rpm. In each flask the quantity of cultivated inoculums was 25 ml. Processes of phenol degradation have been carried out with different media – four experiments with poor media and four experiments with rich media. We used also different initial substrate concentrations of

phenol: 0,02 g dm^{-3}; 0,04 g dm^{-3}; 0,06 g dm^{-3} and 0,08 g dm^{-3}. The phenol degradation time was 10 days.

2.4. Analytical methods

2.4.1. Biomass

The biomass in the samples was determined photometrically by its optical density at wavelength λ = 610 nm on a Specol spectrophotometer (Carl Zeiss, Jena, Germany).

2.4.2. Phenol concentration

Phenol was determined photometrically by a standard colorimetric method based on the formation of a red-colored compound by treating the phenol-containing sample with 3.5 % 4 aminoantipyrine, 20 % ammonium persulfate at the presence of buffer (50 g of NH_4Cl in 900 ml of distilled water), with pH adjusted by ammonium hydroxide to 9,3. The sample was centrifuged and then treated with the above mentioned reagents, and after 15 min, the sample absorbance was measured at wavelength λ= 540 nm. The concentration of phenol was calculated from a calibration straight line.

3. RESULTS AND DISCUSSION

3.1. Biodegradation with poor medium

The experimental results as time evolution of biomass, phenol and added phenol are presented in Fig. 2-5. The experimental results with 0.02 g dm^{-3} initial phenol concentration are shown in Fig. 2.

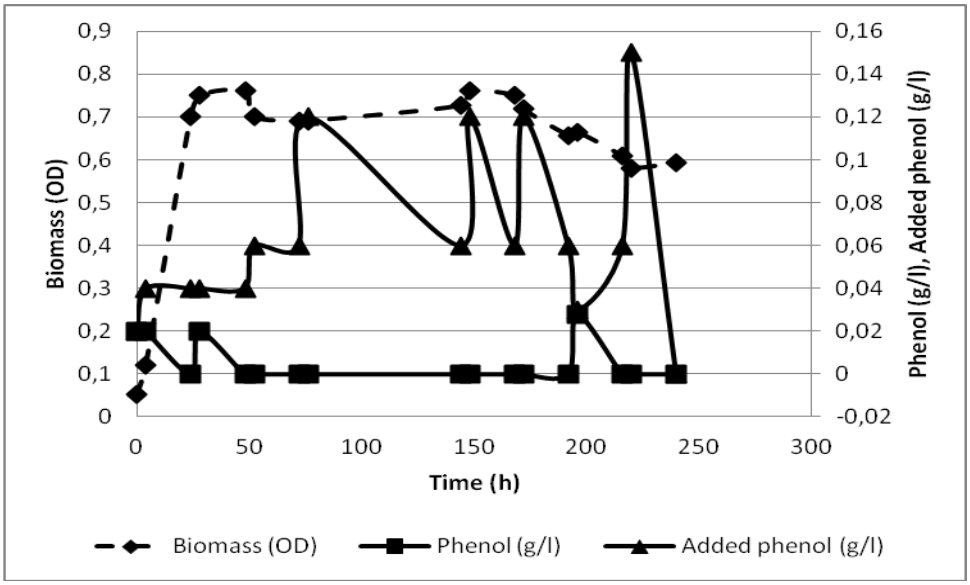


Fig. 2. Curves of biomass growth (dashed line), phenol concentration and concentration of added phenol (solid lines) with 0.02 g dm^{-3} initial phenol concentration

The curve of biomass growth shows a clear and steep exponential-phase which continues to the 28th hour. Then there is a slight decrease in cell growth up to 52th hour. From 52th to 172th hour there is a stable stationary phase, which corresponds to the complete degradation of the phenol over this period. After 172th hour, a decrease in cell growth was observed, which is indicative of the completion of biodegradation.

On the same figure the curves of phenol concentration and the concentration of added phenol are presented. The initial amount of phenol was degraded in 28th hour. This is followed by a series of phenol addition, which lasts up to 200th hour. The maximum amount of phenol added is at 220th hour

and is 0.15 g dm^{-3}. It is degrade in 20 hours. At this initial phenol concentration of 0.02 g dm^{-3}, the total amount of degraded phenol is 1.08 g.

The experimental results with 0.04 g dm^{-3} initial phenol concentration are shown in Fig. 3.

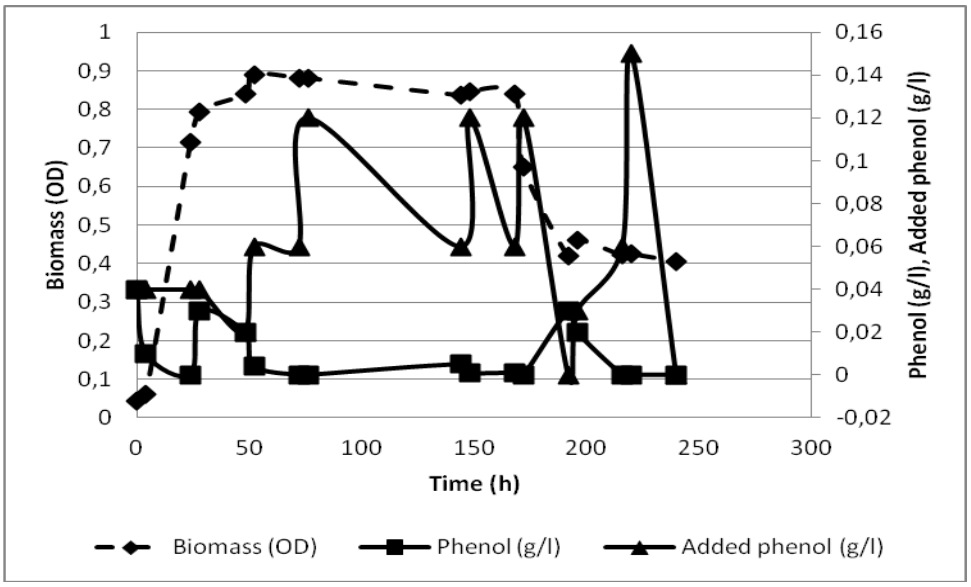


Fig. 3. Curves of biomass growth (dashed line), phenol concentration and concentration of added phenol (solid lines) with 0.04 g dm^{-3} initial phenol concentration

The curve of biomass growth shows a clear and steep exponential-phase which continues to the 28th hour, which is the same as in the previous experiment. The stationary phase then followed up to 168th hour. The difference with the previous experiment is that there is a sharp decrease in biomass growth here, which however does not prevent the degradation of phenol. This shows that even with less biomass, phenol is degraded.

The graph of added phenol shows that there are several peaks of phenol addition: at 76th hour - 0.12 g dm^{-3}; at 148th hour - 0.12 g dm^{-3} and at 220th hour - 0.15 g dm^{-3}. The last amount of phenol added (0.15 g dm^{-3}) was absorbed in 20 hours. The total amount of degraded phenol at this initial concentration of phenol is 1.02 g.

The experimental results with 0.06 g dm^{-3} initial phenol concentration are shown in Fig. 4.

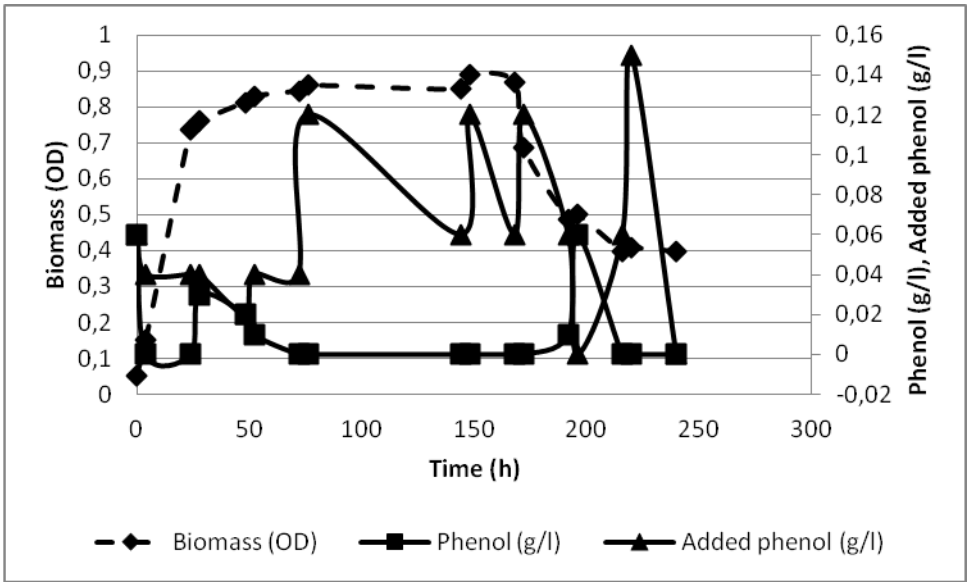


Fig. 4. Curves of biomass growth (dashed line), phenol concentration and concentration of added phenol (solid lines) with 0.06 g dm^{-3} initial phenol concentration

The curve of biomass growth is the same as that of the previous experiment. The difference here is that as biomass grows, the phenol curve shows zero values. That`s why in this experiment, the addition of phenol started as early as the fourth hour. Here again the phenol peaks added were at 76^{th}, 148^{th}, 172^{th} hours with 0.12 g dm^{-3} phenol and at 220^{th} hour with 0.15 g dm^{-3}. The total amount of degraded phenol at this concentration is 1.03 g., which is comparable to that of the experiment with 0.04 g dm^{-3} initial concentration of phenol. This indicates that even with a larger initial amount of phenol, the result is the same as that of the smaller, i.e. *Bradyrhizobium japonicum* 273cells work in the same way.

The experimental results with 0.08 g dm^{-3} initial phenol concentration are shown in Fig. 5.

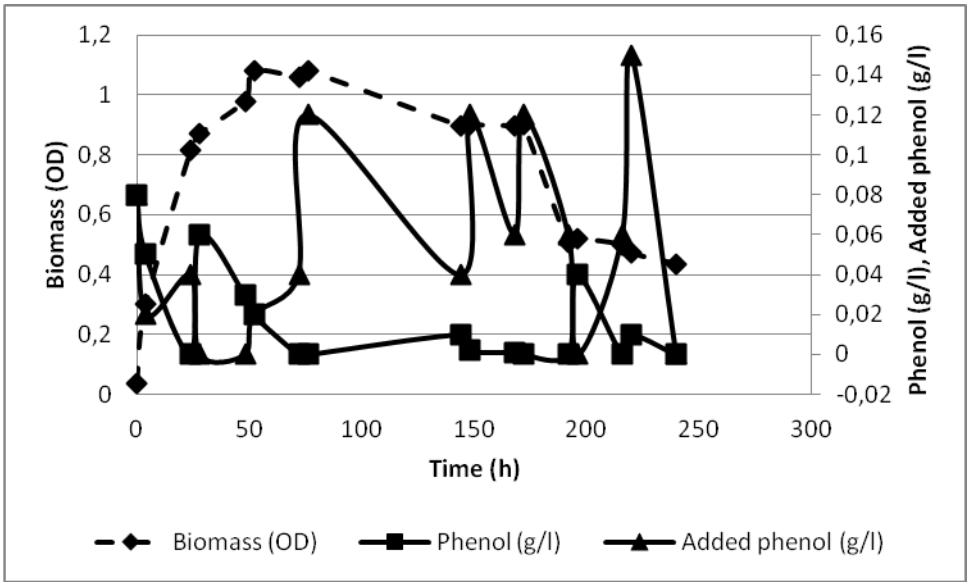


Fig. 5. Curves of biomass growth (dashed line), phenol concentration and concentration of added phenol (solid lines) with 0.08 g dm^{-3} initial phenol concentration

The curve of biomass growth shows a clear exponential phase that lasts up to 52th hour. This is followed by an unstable stationary phase up to 172th hour and a sharp decline in biomass up to 240th hour. In this experiment there was a rather long degradation time of the initial phenol content of 0.08 g dm^{-3}, i.e. the adaptation time is longer. The first addition of phenol is only at 52th hour. In order to avoid substrate inhibition, and given that 0.08 g dm^{-3} initial concentration of phenol is a rather high concentration, it is evident that we have added phenol in much smaller portions here. Peaks of added phenol were again at 76th, 148th, 172th and 220th hour. For the rest of the time, the added amounts are 0.02 and 0.04 g dm^{-3}. This is the reason that the total amount of degraded phenol in this experiment is only 0.93 g. These results indicate that at this high initial phenol concentration *Bradyrhizobium japonicum* 273 cells degrade much less phenol.

3.2. Biodegradation with rich medium

The experimental results for the evolution of biomass, phenol and added phenol are presented in Fig. 6-9.

The experimental results with 0.02 g dm^{-3} initial phenol concentration are shown in Fig. 6.

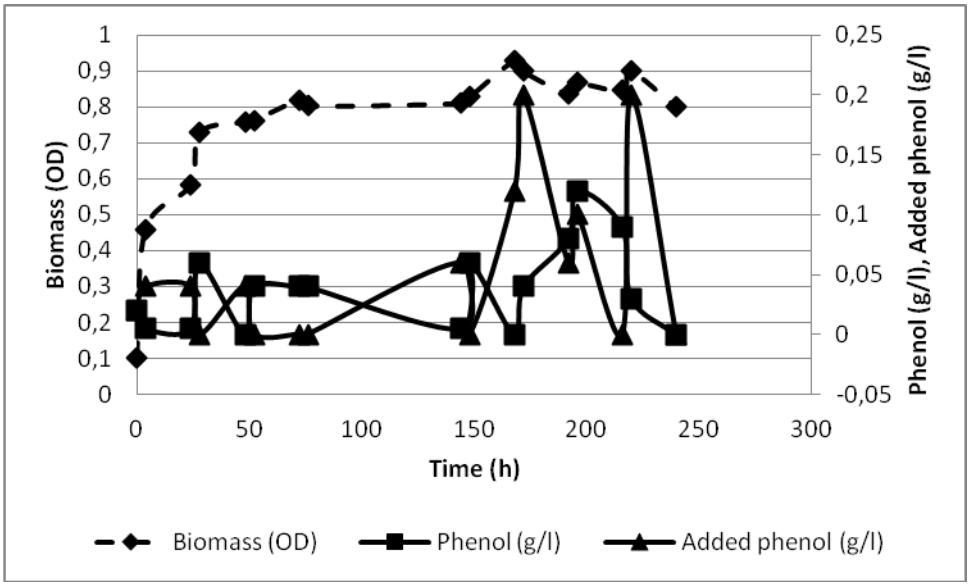


Fig. 6. Curves of biomass growth (dashed line), phenol concentration and concentration of added phenol (solid lines) with 0.02 g dm^{-3} initial phenol concentration

The curve of biomass growth shows weak lag-phase from 4th to 28th hour, a weak exponential phase up to 76th hour and a stationary phase up to 240th hour. Unlike the previous group of experiments, there is no decrease in biomass growth here, but on the contrary it shows a stable stationary phase up to 10 days. The phenol concentration graph shows stable values of 0.04, 0.06 and 0.08 g dm^{-3}. The presence of an additional carbon source in the medium is the reason why small amounts of phenol are added. At 4th, 24th and 48th hour, 0.04 g dm^{-3} phenol was added, and at 148th and 192th hour - 0.06 g dm^{-3}. There are only two peaks of added phenol at 172th hour and at 220th respectively - 0.2 g dm^{-3}. At an initial phenol concentration of 0.02 g dm^{-3} totally 0.88 g. phenol was degraded, which is 0.2 g less than at the same experiment in the first group.

The experimental results with 0.04 g dm^{-3} initial phenol concentration are shown in Fig. 7.

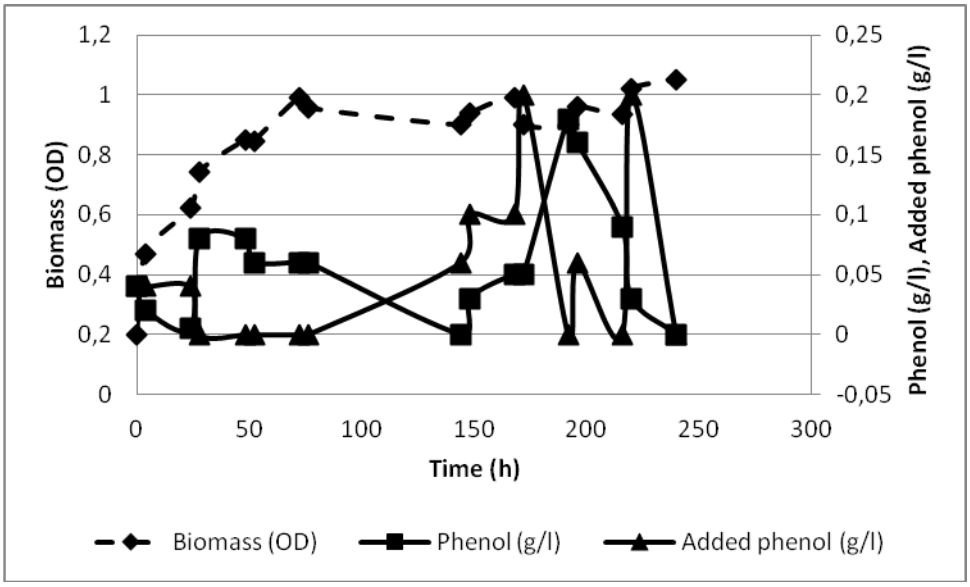


Fig. 7. Curves of biomass growth (dashed line), phenol concentration and concentration of added phenol (solid lines) with 0.04 g dm^{-3} initial phenol concentration

The curve of biomass growth shows a stable exponential phase from 4th hour to 76th hour, followed by a stationary phase with a tendency to increase cell growth. During the exponential phase, the concentration of phenol in the medium is 0.08 g dm^{-3}. This is quite a large amount and due to the fact that during this period the cells absorb glucose, not phenol. After 144th hour, phenol addition of 0.06, 0.1 and 0.2 g dm^{-3} was started. Peaks of added phenol are at 168th, 192th and 220th hour. The total amount of degraded phenol in this experiment is 0.84 g. which is 0.18 g. less than the same experiment in the first group.

The experimental results with 0.06 g dm^{-3} initial phenol concentration are shown in Fig. 8.

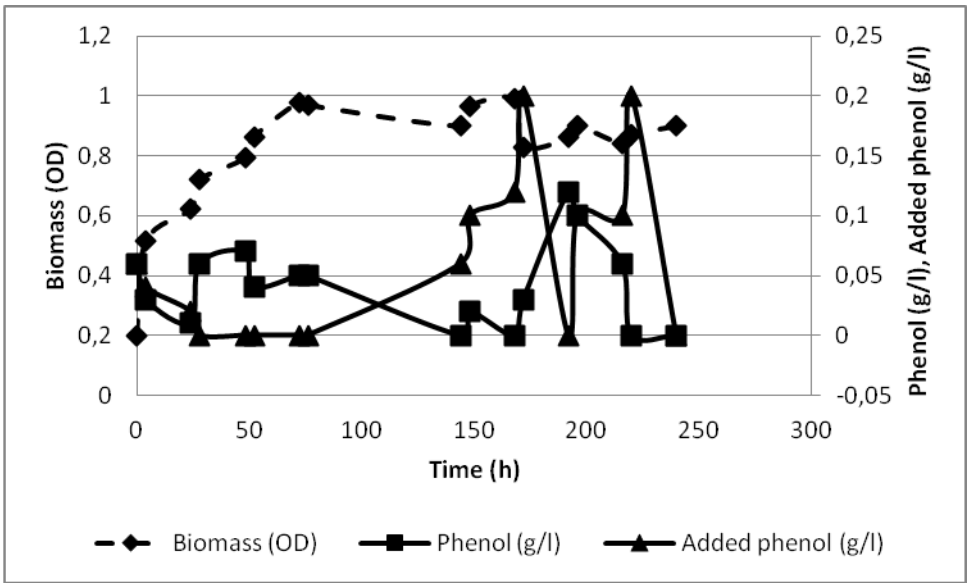


Fig. 8. Curves of biomass growth (dashed line), phenol concentration and concentration of added phenol (solid lines) with 0.06 g dm^{-3} initial phenol concentration

The biomass graph is the same as in the previous experiment of 0.04 g dm^{-3} initial phenol concentration. A stable exponential phase of 4th hour to 76th hour is seen, followed by a stationary phase with a tendency to increase cell growth. The graph of phenol concentration indicates that there is also difficulty in absorbing phenol here. From 0th hour to 144th hour the biomass grows from the starting phenol 0.06 g dm^{-3} and from the second carbon source (glucose). This is the reason that we add phenol only after 144th hour. The maximum amounts of phenol added are 0.2 g dm^{-3} at 172th hour and at 220th hour respectively. The total amount of degraded phenol in this experiment is 1 g. This is 0,03 g less than the same experiment in the first group. The reason for the lower absorption of phenol is the presence of an additional carbon source. The difference is the smallest so far because the initial concentration of phenol is greater than the other experiments.

The experimental results with 0.08 g dm^{-3} initial phenol concentration are shown in Fig. 9.

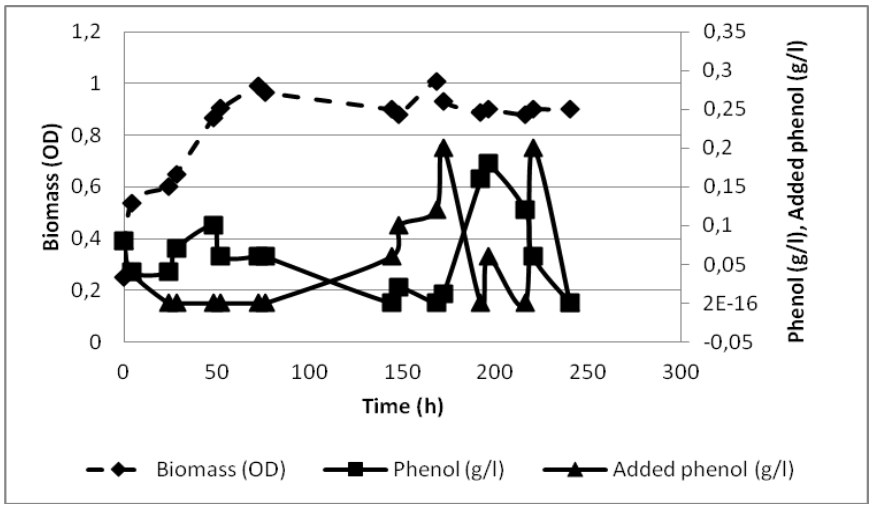


Fig. 9. Curves of biomass growth (dashed line), phenol concentration and concentration of added phenol (solid lines) with 0.08 g dm^{-3} initial phenol concentration

From the biomass graph, one clearly expressed exponential phase is visible, followed by a stable stationary phase from 72th to 240th hour. No phenol is added for 76 hours. The reason for this is the high initial concentration of phenol, which is more difficult to degrade, as well as the presence of glucose. The amount of phenol in the medium is 0.18 g dm^{-3}, which degrades to 220 hours. After the initial phenol and glucose were depleted for 144 hours, we began to add phenol. The maximum amount of phenol added is 0.2 g dm^{-3} at 172th hour and at 220th hour respectively. The graph of phenol concentration shows that at 196th hour the degradation of phenol, added at 172th hour, is difficult. The total amount of degraded phenol in this experiment is 0.86 g which is 0.07 g less than the same experiment in the first group and this is due to the fact that there is a greater amount of phenol in the initial concentration of the substrate.

CONCLUSIONS

From the presented experimental results of phenol biodegradation, the following conclusions can be drawn:

1. The maximum amount of degraded phenol is 1.08 g. It was obtained in an experiment with 0.02 g dm^{-3} initial phenol concentration when working in poor medium.
2. The strain *Bradyrhizobium japonicum 273* cells hardily degrades phenol with initial concentrations of 0.06 and 0.08 g dm^{-3} in both media.

3. When using a poor nutrient medium, the amount of degraded phenol is greater than that in the rich nutrient medium.
4. In experiments with a rich medium, *Bradyrhizobium japonicum 273* cells grow thanks to the second carbon source, i.e. glucose, which causes a smaller amount of phenol to be absorbed.

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