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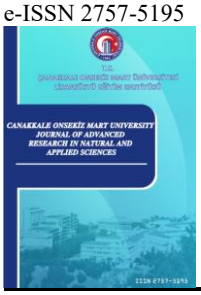

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Effect of Grovth Medium on L-Dopa and Dopamine Production Using *Citrobacter freundii* (NRRL B-2643)

Meltem Çakmak[1], Veyis Selen[2], Dursun Özer[3], Fikret Karataş[4*], Sinan Saydam[5]

[1,3] Department of Chemical Engineering, Faculty of Engineering, Fırat University, Elazığ, Türkiye
[2] Department of Bioengineering, Faculty of Engineering, Fırat University, Elazığ, Türkiye
[4,5] Departmen of Chemistry, Faculty of Science, Fırat University, Elazığ, Türkiye

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Research Article

Abstract – In this study, microbial production of L-Dopa and Dopamine which is an important substrance for the treatment of Parkinson's disease were investigated by using *Citrobacter freundii* (NRRL B-2643). The effects of carbon source (sucrose) and, salt concentrations (NaCl, $CaCl_2$), initial pH, temperature, inoculum level and shaking speed on L-Dopa and dopamine production were investigated. The amounts of extracellular L-dopa and dopamine were determined by using HPLC. Maximum L-dopa and dopamine production, under optimized conditions (sucrose: 2.5 g/L, NaCl and $CaCl_2$: 1.0 g/L, inoculum level: 1.0% (v/v), initial pH: 6.5, temperature: 33°C, shaking speed: 200 rpm) were found to be 458 and 592 mg/L, respectively. Although the experiments were carried out for 60 hours, but the maximum production of L-Dopa and Dopamine was realized at around the 30th hour of the experiments.

Keywords – *Citrobacter, dopamine, fermentation, freundii (NRRL B-2643), L-Dopa, HPLC*

1. Introduction

Although there are millions of living species in nature, there are common structures and characters among living things is divers. Microorganisms have many genes that humans have, as well as some genes that are not to be found in humans.

Bacteria use cheap carbon sources to reproduce, have the ability to reproduce quickly, and can be modified due to their relatively simple structure. Bacteria are used in the natural synthesis of many drugs used in the health industry, as they provide cheap cost due to these properties (Demain & Vaishnav, 2009). Bacteria is a microscopic single-cell species that prosper in a number of medium, such as soil, water, and can be grouped on the basis of cell structure, cell metabolism or cell component variations such as DNA, fatty acids, pigments, antigens and quinones. *C. freundii* is a member of the Enterobacteriaceae family of anaerobic gram-negative bacteria (O'Hara et al. 1997; Fang et al. 2017).

L-Dopa (3,4-dihydroxy-L-phenylalanine or 2-amino-3-(3,4 dihydroxyphenyl)-propanoic acid) is an amino acid naturally synthesized in the human body from L-tyrosine. L-Dopa is formed as a result of hydroxylation of tyrosine by the tyrosine hydroxylase (TOH) and leads to the biosynthesis of catecholamines such as dopamine, norepinephrine and epinephrine, which have an important biological functions (Nagatsu & Sawada, 2009; Kurt et al. 2009).

[1] cakmak_meltem@hotmail.com
[2] vselen@firat.edu.tr
[3] dozer@firat.edu.tr
[4] fkaratas@firat.edu.tr
[5] ssaydam@firat.edu.tr
[*]Corresponding Author

L-Dopa is used as a primary drug in the treatment of Parkinson's disease and neurogenic injury following the myocardium (heart muscle) (Raju & Ayyanna, 1993). Unlike dopamine, the chirality of L-Dopa enables it to selectively cross the blood-brain barrier to the brain. Therefore, L-Dopa is used as the most effective drug in the treatment of Parkinson's patients (Montgomer, 1992). In recent years, in addition to being a dopamine precursor, L-Dopa has been reported to act as a neuromodulator or neurotransmitter in the central nervous system (Misu et al. 2002). Dopamine is the precursor of the mammalian central nervous system neurotransmitters norepinephrine and epinephrine (Kruk & Pycock, 1991). Dopamine is synthesized from L-Dopa by the enzyme dopa decarboxylase (DDC; aromatic L-amino acid decarboxylase, EC 4.1.1.28). Dopamine is a neurotransmitter that is the starting material for the formation of important catecholamines (such as norepinephrine and epinephrine) and also activates dopamine receptors in the brain (Hoffman &Lefkowitz, 1996; Lisbon, 2003). Dopamine synthesis takes place in two stages. In the first stage, L-Dopa is synthesized from substrates such as tyrosine and catechol, while Dopamine is synthesized from L-Dopa in the second stage. While a bacterium (*Symbiobacterium sp*) was needed for the synthesis of L-Dopa, another bacteria was needed for the synthesis of Dopamine (*Streptoccus faecalis*) from L-Dopa (Lee et al. 1999).

Bacteria can reach large masses in suitable nutrient media due to the the highest reproduction rate, the lowest generation time, and they show a logarithmic growth. Many products with high potential for use in various industries are obtained from microorganisms using recombinant techniques. Especially the use of bacteria for the production of drugs used in the treatment of many diseases is of great importance for the pharmaceutical industry.

In this study, simultaneous production of Dopa and dopamine was carried out by using the *Citrobacter freundii* (NRRL B-2643) bacteria. Aimed was to optimize the fermentation conditions (such as sucrose concentration, pH, temperature) for the production of L-Dopa and dopamine.

2. Materials and Methods

2.1. Material

C. freundii (NRRL B-2643) microorganism was obtained from the Biotechnology Laboratory of the Chemical Engineering Department of Fırat University. Double distilled (dd H_2O) water was used throughout the work. All the chemical used are reagent or analytical grade and obtained from Merck or Sigma-Aldrich.

2.2. Method

Luria-Bertani (LB) medium was used for the storage and development of *C. freundii* microorganism used in experiments (Bertani, 1951). The prepared stock cultures were cultivated in solid nutrient medium (10.0 g peptone, 5.0 g yeast extract, 10.0 g NaCl and 15 g agar, per liter) and incubated at 37 °C for 18 hours. The culture obtained after incubation was kept at +4 °C as it was used in a short time. Before the bacteria required for the production of L-Dopa and Dopamine are inoculated into the prepared fermentation medium, they are inoculated from the culture storage medium to the development medium and then inoculated into the main fermentation medium. Thus, the fermentation medium is inoculated with fresh culture.

50 ml of the nutrient media (LB) prepared for the experiments were taken and production was carried out in 250 mL erlen mayer after sterilization. The erlen mayers, in which the cultures were inoculated, were placed in an orbital shaker (Selecta Rotabit) with the shaking speed of 150 rpm at 37 °C and incubated for 18 hours to ensure bacterial growth. At the end of the incubation, the microorganisms whose growth was completed were transferred to the L-Dopa and Dopamine production medium at a different amount. L-Dopa and dopamine analysis were performed by taking a sample one erlen mayer from orbital shaker for every 6 hours, and centrifuged at 6000 rpm for 5 minutes then supernatant is separated. Experiments were carried out in three parallels.

2.3. L-Dopa and Dopamine Analysis

The amounts of extracellular L-Dopa and Dopamine in the supernatant were determined by HPLC (Shimadzu LC20). L-Dopa and Dopamine were determined at 290 nm by using a mixture of 97/3 (v/v) 25 mM Phosphate Buffer (pH:3.0) and Methanol as the mobile phase in an Inertsil ODS-4 column at a flow rate of 1.0 mL/min. (Aytan E. 2009)

3. Results and Discussion

The effects of sucrose concentration, initial pH, temperature, shake speed and salt concentration (NaCl vs. $CaCl_2$) on L-Dopa and Dopamine production by *C. freundii* (NRRL B-2643) were investigated. The composition of the medium is the most important parameter affecting the growth of microorganisms and the production of various metabolites. The carbon source is very important as it participates in the synthesis of basic substances that form the building blocks of the microorganism cell, such as polysaccharides, lipids and proteins, and is also used as an energy source (Rehm et al. 1987). Different concentrations of sucrose (1-10 g/L) were used to investigate the effect of carbon source on L-Dopa and Dopamine production. The results obtained are given in Figures 1 and 2.

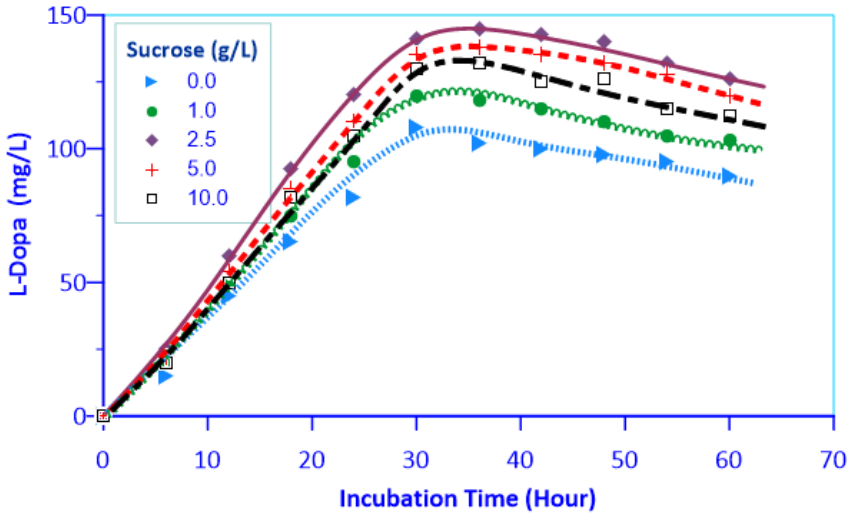

Figure 1. The effect of sucrose concentration on the L-Dopa production

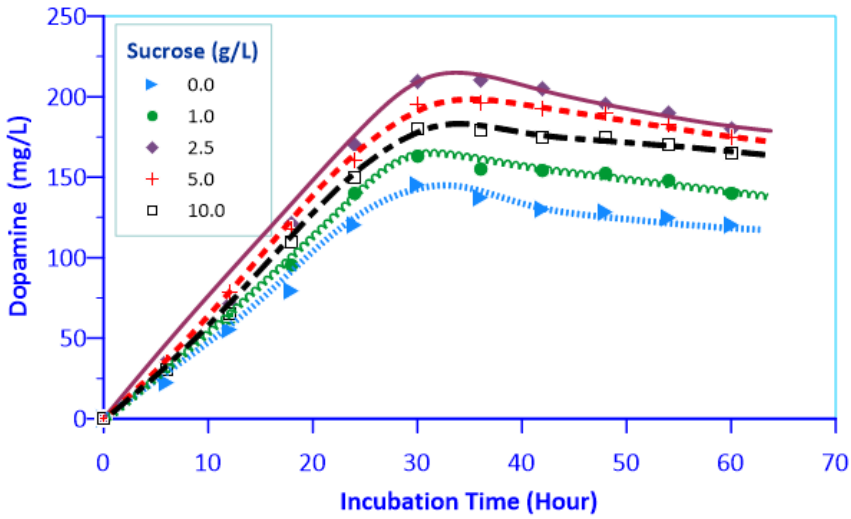

Figure 2. The effect of sucrose concentration on the Dopamine production

As seen in Figures 1 and 2, the highest L-Dopa and Dopamine concentrations were reached at 2.5 g/L sucrose concentration, 141 and 209 mg/L respectively.

It is seen that high sucrose concentration has a negative effect on L-Dopa and Dopamine production and decreases the production level of these metabolites. The reason for this is that the presence of some carbon sources in high concentrations in the nutrient medium reduces the amount of substances produced by making a repression effect on the metabolites secreted by microorganisms (Prudende et al. 1989; Yun & Ryu, 2001; Geckil et al. 2004).

Towards the end of the logarithmic phase, it is seen that the production amount L-dopa and dopamine's are almost fixed, indicating that the production is accompanied by growth. In other words, growth associated products are produced simultaneously with microbial growth.

The decrease in the amount of products towards the end of the incubation period indicates that the products started to deteriorate due to the environmental conditions. Production of L-dopa and dopamine is stopped at the 30 th hour to prevent product loss. Since L-Dopa is the precursor of Dopamine, the variation of product concentrations over time is expected to be parallel (Figures 1 and 2).

Optimum nitrogen source for the production of L-Dopa and Dopamine with the C. *fruendii* microorganism were used according to Çakmak, (2016), 10.0 g/L peptone, 5.0 g/L yeast extract, 10.0 g/L NaCl, initial pH: medium pH, inoculum level: 1.0 % v/v, shaking speed: 150 rpm, T: 37 °C. In addition to carbon and nitrogen sources, microorganism needs elements such as Na, Ca, Mg, Fe, Mn, Cu, Zn in order to grow and produce products. Trace elements are sometimes especially added to the growth medium, but it is generally obtained through impurities in water and other media components. These cations are usually obtained from inorganic salts. In addition to their nutritional role, these salts also have a stimulating role on development of microorganism and activate enzymes, in some cases it is a component of some enzymes (Shuler & Kargi, 2002). In order to investigate the effect of Sodium and Calcium, chloride salts were added to the fermentation medium. It was observed that concentrations of NaCl had no effect on the amount of product, but $CaCl_2$ concentrations had a slightly positive effect on the production of L-dopa and dopamine concentration. It is known that NaCl creates an isotonic balance in fermentation environments (Halkman, 2005). In the continuation of the study, NaCl was added to the fermentation medium at a concentration of 1.0 g/L. In order to investigate the effect of calcium, $CaCl_2$ was added to the fermentation medium at five different concentrations, 1, 2.5, 5, 7.5 and 10 g/L. The effect of $CaCl_2$ on L-Dopa and dopamine production is given in Figures 3 and 4.

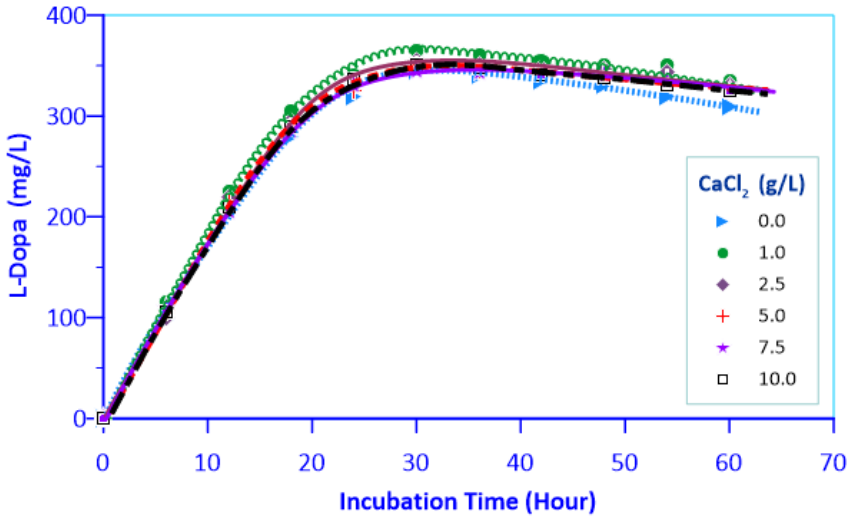

Figure 3. The effect of $CaCl_2$ concentration on the L-Dopa production

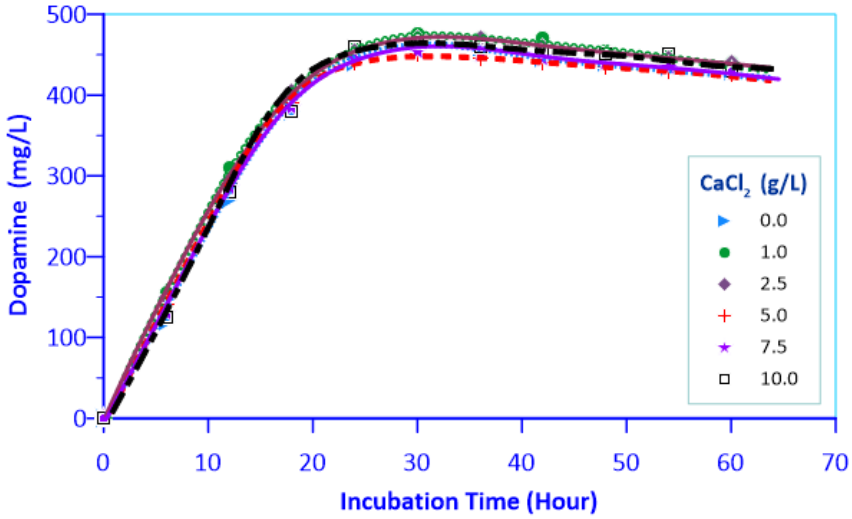

Figure 4. The effect of $CaCl_2$ concentration on the Dopamine production

As seen in Figures 3 and 4, the highest L-Dopa and Dopamine amounts were obtained at 1.0 g/L $CaCl_2$ concentration. At this concentration, the amounts of L-Dopa and dopamine were found to be 363 and 471 mg/L, respectively. Since the isotonic balance in the medium is largely achieved with NaCl, the amount of $CaCl_2$ should be well adjusted. Calcium, helps to regulate the ionic balance, and it also acts to activate some enzymes in the medium (Halkman, 2005).

In the microbial production processes, the pH of the medium changes over time due to the conversion of the carbon sources used into different products, the formation of some intermediate organic acids and their reuse to produce other compounds. pH is very important for microorganism growth and production product (Shuler & Kargi, 2002). The results obtained from the experiments carried out to investigate the effect of initial pH on L-dopa and dopamine production are given in Figures 5 and 6.

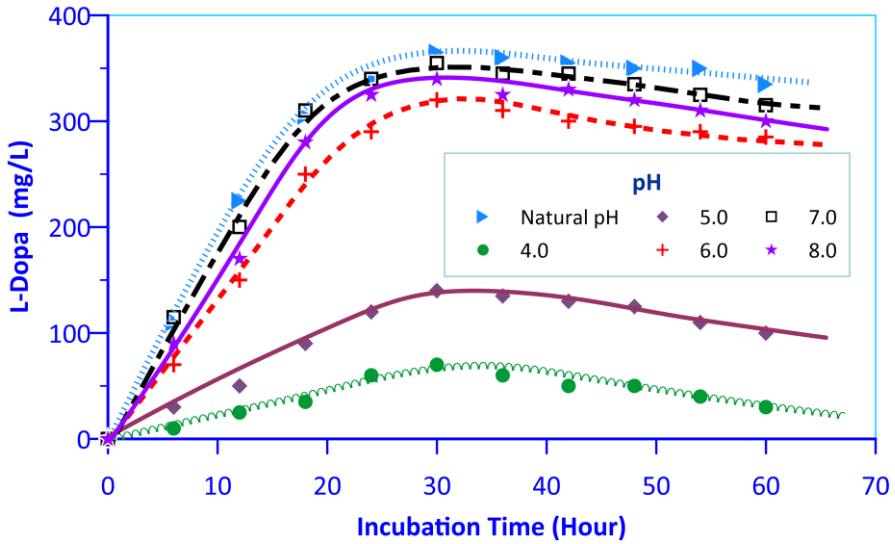

Figure 5. Effect of initial pH on L-Dopa production

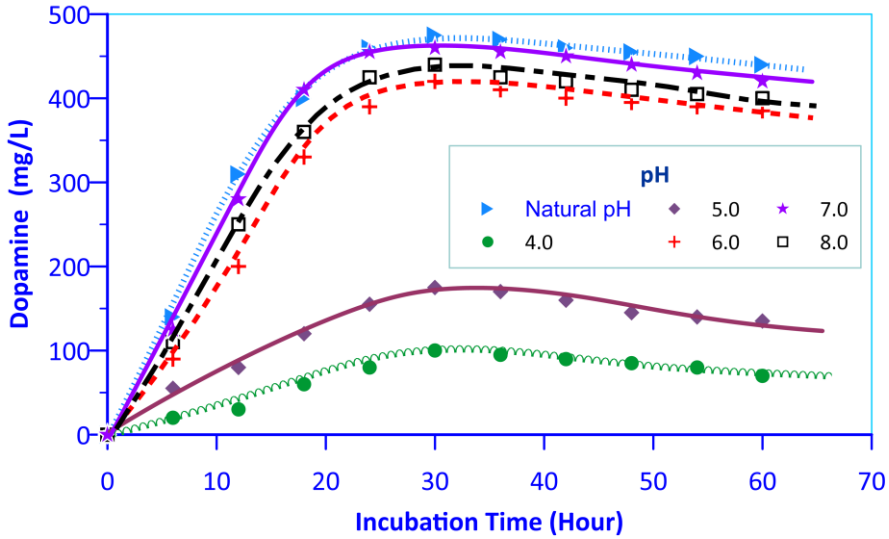

Figure 6. Effect of initial pH on Dopamine production

Maximum production of L-Dopa and Dopamine occurred at the natural pH of the medium (approximately 6.5). The amounts of L-Dopa and dopamine formed at this pH were found to be 363 and 471 mg/L, respectively (Figures 5 and 6). At low pH values, production of L-dopa and dopamine might be reduced, because of low microorganism production at this pH. Apart from natural and near-nature pH values, L-Dopa and dopamine production are adversely affected. Elibol and Ozer (2000) reported in their study that the amount of lipase produced varies depending on the initial pH. Our findings are compatible with the literature.

It is reported that the amount of inoculum affects microbial processes (Elibol et al. 1995). The results obtained in the study conducted to investigate the effect of different inoculum level on L-Dopa and Dopamine production are given in Figures 7 and 8.

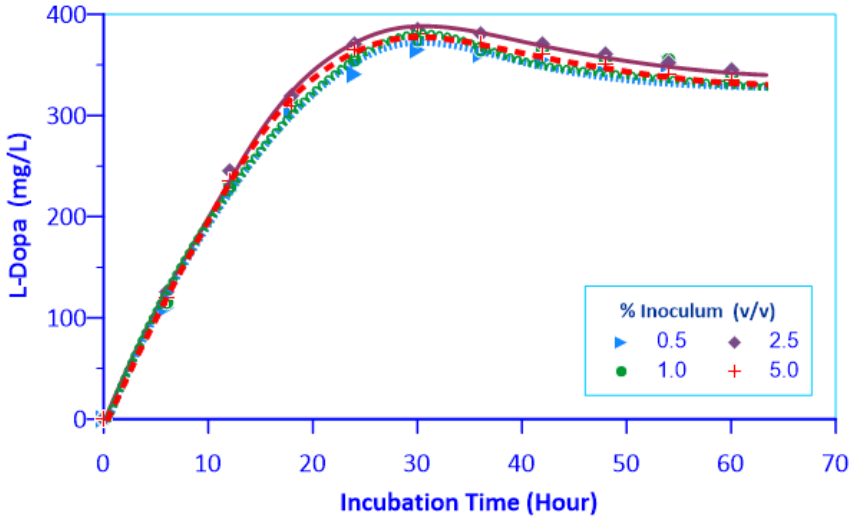

Figure 7. Effect of inoculum level on L-Dopa production

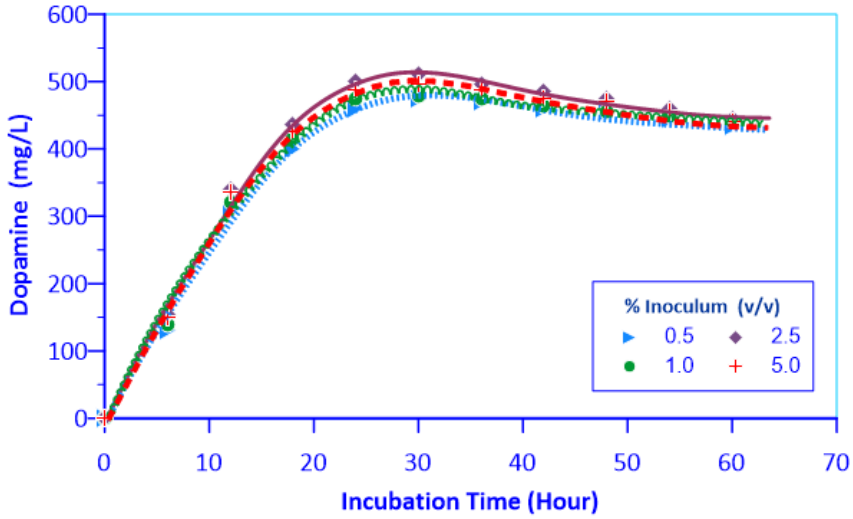

Figure 8. Effect of inoculum level on Dopamine production

As seen in Figures 7 and 8, although the increase in inoculum concentration did not affect L-Dopa and Dopamine production much, the highest production level was reached in the medium where microorganisms were cultivated at a ratio of 2.5% by volume. These values are 382 and 511 mg/L, respectively. Elibol and Özer (2000) reported in their study that the amount of inoculum had no effect on the amount of lipase produced.

Mixing is a very important parameter for microbial growth and production, as it facilitates the microorganism's access to oxygen and nutrients (McDaniel & Bailey, 1969).

The effect of shaking speed on L-dopa and dopamine production is given in Figures 9 and 10.

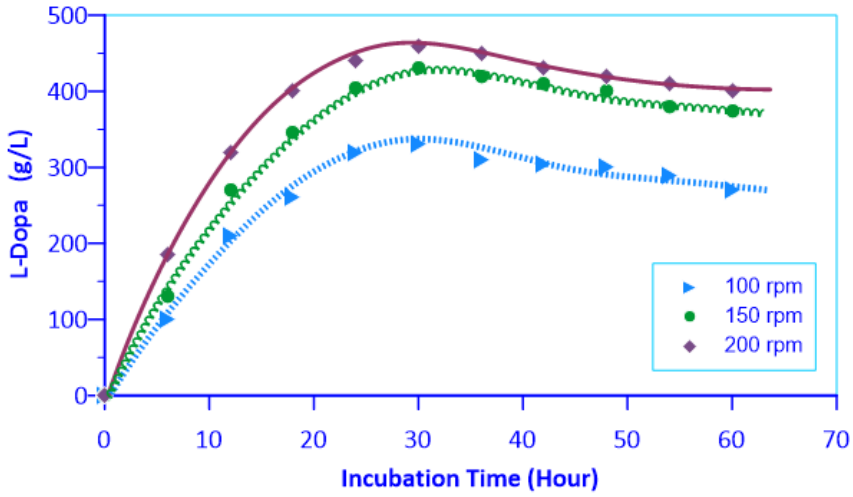

Figure 9. Effect of shaking speed on L-Dopa production

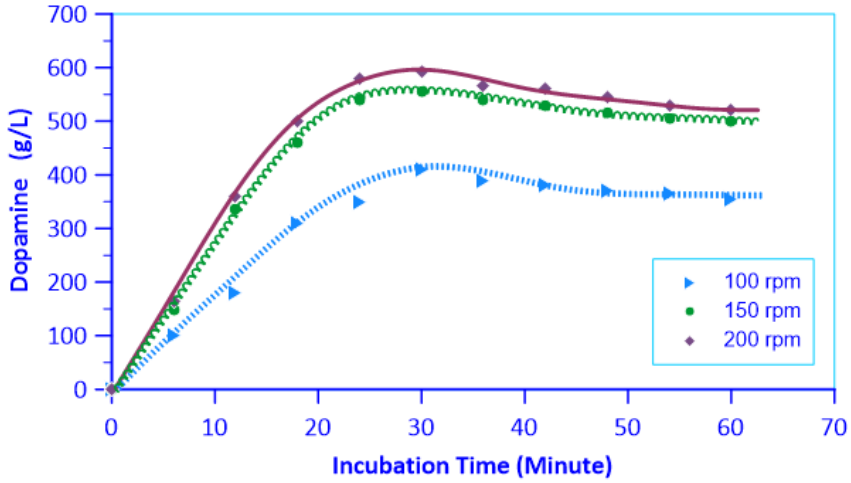

Figure 10. Effect of shaking speed on Dopamine production

It is seen that the amount of product increases depending on the shaking speed. As the film layer around the microorganism cells will be thinned with mixing, the oxygen and nutrient transfer to the cells will increase. If the shaking speed is too high, this create a disadvantage for microorganisms, the speed must be adjusted well. Charm and Wong (1970) reported that high mixing speeds negatively affect the activity of enzymes. Temperature is an important parameter in fermentation processes and needs to be adjusted well. Higher temperature increases cell denaturation and has a negative effect on cell formation. In addition, at high temperature values, the structure of the formed products may deteriorate with the deterioration of microorganism proteins. Since the diffusion of nutrients to the microorganism is reduced at low temperature values, the microorganism cannot develop sufficiently and the production amount decreases (Sodhi et al. 2005; Prakasham et al. 2005).

In order to investigate the effect of temperature on L-Dopa and Dopamine production, the results of experiments performed at different temperatures are given in Figures 11 and 12.

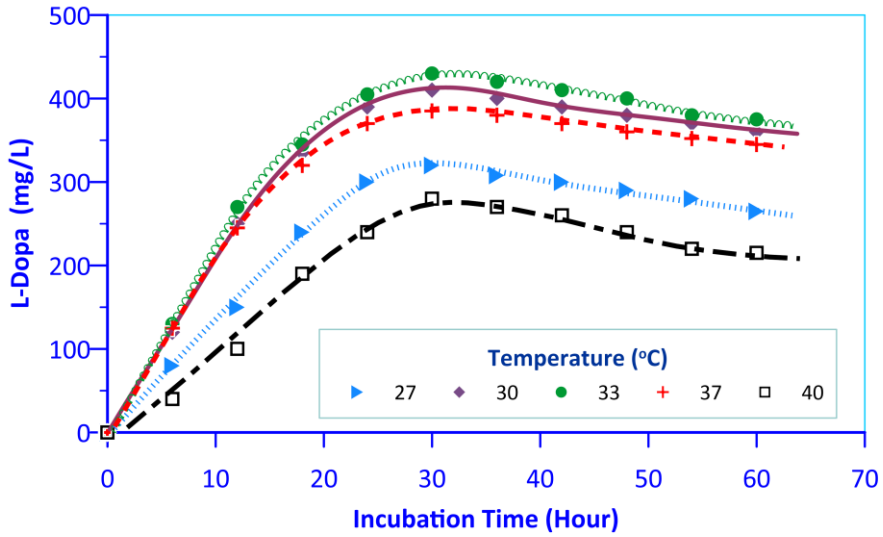

Figure 11. Effect of temperature on L- Dopa production

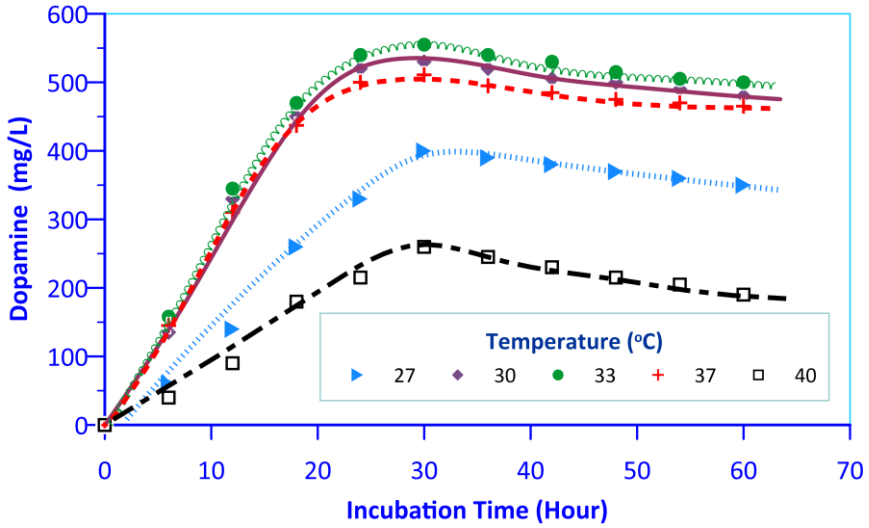

Figure 12. Effect of temperature on Dopamine production

The highest production of L-Dopa and Dopamine occurred at 33 °C. The amounts of L-Dopa and dopamine formed at this temperature are 428 and 553 mg/L, respectively. It is observed that at higher temperatures the amount of L-dopa and dopamine concentration are decreased (Figures 11 and 12). Tanyildizi et al. (2007) reported that amylase production decreased at high temperatures.

4. Conclusion

It has been determined that especially sucrose concentration, temperature, initial pH and mixing speed are very effective on the production of L-Dopa and Dopamine by fermentation. It has been observed that the production of L-Dopa and dopamine reaches its maximum in approximately 30 hours. Extending the fermentation time caused a slight decrease in the amount of both products. Maximum production conditions for L-Dopa and Dopamine were determined to be 2.5 g/L sucrose, 1.0 g/L $CaCl_2$, 2.5% (v/v) inoculum, 33 °C temperature and initial natural pH. To increase the production of dopa and dopamine at these conditions, different genetically modified microorganism could be used for further studies. In addition, L-dopa and dopamine production could be investigated by batch and continuous reactors.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Meltem Çakmak: Performing the experiments.
Veyis Selen: Performing the experiments.
Dursun Özer: Designing the experiments, evaluating the results and writing.
Fikret Karatas: Designing the experiments, evaluating the results and writing.
Sinan Saydam: Designing and writing the manuscript.

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