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Optimization of medium composition for the production of peroxidase by *Bacillus* sp.

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ABSTRACT

The bacterium, Bacillus sp. holds good promise as a biotreatment tool due to its ability to produce the peroxidase enzyme which has the potential to degrade various hazardous compounds. In this article, we describe the selection of medium components like pH, temperature, carbon sources, nitrogen sources and amino acids for the optimal production of peroxidase by Bacillus sp. with a series of experiments which is important for peroxidase production. Peroxidase enzyme find useful in a variety of biotechnological fields such as pulp and paper, laundry and textile industries, for the decomposition of pollutants. It can be further used in many newer areas where they can serve as potential biocatalysts.

Keywords: Peroxidase, Production, *Bacillus* sp., Optimization

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INTRODUCTION

Peroxidases are widely found in throughout the plants, animals and microorganisms, signifying their key role in biological systems. It oxidizes a large number of low molecular weight aromatic compounds like hydroquinones, phenols, flavonoids, benzidine derivatives, and macromolecules of lignin or humic substances. Peroxidase has attracted industrial attention because of its usefulness as a catalyst in the pulp and paper, textile and laundry industries, for the decomposition of pollutants, or for use as biosensors and other applications [1, 2]. The well known peroxidase is that from horseradish root which due to its wide specificity for H_2 donors and its superior catalytic efficiencies has been used extensively in spectrophotometric determinations of biological resources.

Peroxidases are classified as oxidoreductases and they are given the official EC number 1.11.1.7. In peroxidase enzyme the bound cofactor essential for its activity is haem. Haem is a complex between an iron ion and protoporphyrin molecules. Peroxidase catalyzes the transfer of oxygen from the hydrogen peroxide to an appropriate substrate and consequently brings about oxidation of the substrate. Peroxidase use H_2O_2 as electron acceptor for catalyzing various oxidative reactions.

Many reports have been shown the importance of microbial peroxidases in the degradation of various compounds including dyes, olive mill waste waters, nitro aromatics, dioxins, chlorinated and many other compounds as reviewed by Goszezynski et al. [3]. Importantly, peroxidases are widely present in both gram positive and gram negative bacteria. In bacteria peroxidases are found as intracellular enzymes and seemed to play important roles in protection against H_2O_2 induced cell damage. The *Flavobacterium meningosepticum* produces peroxidase during nucleoside metabolism which degrades H_2O_2 [4]. Peroxidases appeared to be involved in the degradative mechanism of azo dyes and they secreted in *Flavobacterium*, *Streptomyces* and *P. chrysosporium* [5]. Until now, peroxidises have been isolated and characterized from various bacteria including *Deinococcus radiophilus* [6], *Escherichia coli* [7], *Septoria tritici* [8], *Bacillus stearothermophilus* [9], *Klebsiella pneumonia* [10], *Rhodobacter capsulatus* [8, 11], *Streptomyces* sp. [12], *Mycobacterium smegmatis* [13], *Mycobacterium tuberculosis* [14, 15] and *Synechocystis* sp.

[16]. Bacillus spp. may also contribute to the fermentation process by their polyphenol oxidase and peroxidise activities [17].

Phenols and anilines are present in number of industrial wastewaters, such as those concerning coal conversion, plastics and resins, organic chemicals and petroleum refining textiles dyes. Most of these phenols and anilines are very toxic materials. Removal of these toxic chemicals from wastewater by means of peroxidases instead of physical and chemical methods has many advantages, such as inexpensive, no formation of hazardous byproducts and high efficiency [18]. In the above fields, many processes are unstable under alkaline conditions and have optimum pHs of below or around pH.6.0. Thus, the findings of alkalophilic peroxidases are imperative for practical purposes.

The objective of this investigation was to determine the optimum conditions of pH, temperature, carbon, nitrogen and aminoacid sources and to assess their effects on the peroxidise production of *Bacillus* sp.

MATERIALS AND METHODS

Organism and culture conditions

Bacillus sp. which obtained from laboratory stock was tested for their potential of producing peroxidase enzyme. Pure culture was maintained on the nutrient agar plate and incubated at 32°C for further experimental work. For preparation of inoculum, *Bacillus* sp. was grown in 100 mL Erlenmeyer flasks containing 25 mL of nutrient broth (NB) medium under shaking conditions (150 rpm) at 32°C for 24 h. To determine peroxidase activity, *Bacillus* sp. was grown in medium containing (g/L): Glucose, 10.0; $NaNO_3$, 2; KH_2PO_4, 1.0;

$MgSO_4.7H_2O$, 1.0; NaCl, 1.8; KCl, 0.5; $CaCl_2.2H_2O$, 0.5g; $FeSO_4$, 0.01; Histidine, 0.5. The pH of this medium was adjusted to 7.6 with NaOH (1.0M).

Optimization of processing parameters

The effect of different parameters on peroxidise production was studied individually, by varying one factor at a time. At each step, the selected factor was included in the control medium for getting a set of conditions that enabled maximum production of peroxidase from *Bacillus* sp. In all cases, samples were taken every 6 h interval and analyzed for peroxidise activity as described below.

Effect of temperature

Bacillus sp. was grown in production medium at varying temperatures ranging from 20°C to 60°C to ascertain its influence on peroxidise production.

Effect of pH

In order to study the effect of initial pH of the culture medium on peroxidise production, the bacteria was grown in the production medium with initial pH varied between 5.0 and 11.

Effect of carbon sources

To check the effect of different carbon sources (glucose, starch, carboxy methyl cellulose (CMC), fructose, mannitol, maltose, lactose, sucrose) on the peroxidase production each of the carbon source selected was used separately in production broth (100 mL) at a concentration of 1 % (w/v). The effect of concentration of best carbon source (mannitol) on the production of bacterial peroxidase was determined by adding 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (w/v) of mannitol to the production broth under above conditions.

Effect nitrogen sources

The effect of various nitrogen sources on the production of peroxidase was studied by adding 0.5% (w/v) of chosen (peptone, beef extract (BE), yeast extract (YE), NH_4Cl, $NaNO_3$,

$(NH)_2HPO_4$, NH_4NO_3) nitrogen sources individually to the production broth. Yeast extract, which was found to be the best organic nitrogen source for peroxidise production, was studied at different levels (0.5% to 3.0%) for understanding the optimum concentration required for maximum peroxidase activity. In addition, the effect of concentration of best inorganic nitrogen source ($NaNO_3$) on the production of peroxidase was determined by adding 0.1, 0.2, 0.3, 0.4 and 0.5% (w/v) of $NaNO_3$ to the production broth under above conditions.

Effect of amino acids

The effect of various amino acid sources on the production of peroxidase was studied by adding 0.2% (w/v) of chosen (glutamine, glycine, aspartic acid, alanine, methionine, phenylalanine, leucine, proline, asparagine, tyrosine) amino acids individually to the production broth.

Determination of peroxidase activity

Culture flasks of *Bacillus* sp. was centrifuged at 12,000g at 4°C for 20 min. The pellets were discarded and the supernatants were collected for determining peroxidase activity. Peroxidase activity was assayed spectrophotometrically. The increase in the absorbance was calculated at 414 nm using 100 mM of citrate phosphate buffer, 1.7mM ABTS (2, 2 azino bis 3 ethyl benzo thiazolin 6 sulphonic acid), 2.5 mM H_2O_2 (these are final assay concentration for 1 mL of reaction mixture). To 0.1 mL of supernatant, 0.9 mL of 1.7 mM ABTS and 25µl of 2.5 mM H_2O_2 was added and the OD was read at 414 nm for one minute. 0.1 mL of distilled water, 0.9 mL of 1.7 mM ABTS and 25 µl of 2.5 mM H_2O_2 were kept as blank. Heat denatured enzyme sample served as control. One unit of peroxidase enzyme was defined as the change in absorbance of 1.0/ml/min at 414 nm.

RESULTS AND DISCUSSION

Recently more attention has been focused on the enzyme peroxidases since they occupy an important position with respect to their applications in both physiological and commercial fields. Peroxidases from animals and plants are generally unable to meet the current demands which have led to an increase in microbial peroxidases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. It can serve as preferred source of peroxidase enzymes because of their fast growth, limited space required for their cultivation and the ease with which they can be genetically manipulated to make new enzymes are desirable for various biotechnological applications. Almost in all processes industrial applications of enzymes being performed at elevated temperature, high or low alkalinity and salt concentrations. These characteristics well go with the abilities of microbes from extreme environments.

Medium pH and temperature

To ascertain the optimum incubation pH and temperature for maximum production of peroxidase, the bacterium was incubated at different pH such as 5 to 11 and at a temperature of 30 to 60°C. These two experiments were conducted separately. The bacterium *Bacillus* sp. was able to release a maximum peroxidase production of 32.1U/mL at pH 8.0 after 18 h and therefore alkaline pH moderately supported the peroxidase production (Fig. 1). Acidic pH did not favour for high enzyme production. Regarding the temperature, highest peroxidase activity was recorded at 30ºC which showed 49U/mL at 18 h (Fig. 2). The catalase-peroxidase from *Bacillus* sp. No. 13 also had a wide optimum pH range, and it was quite stable at high temperature and pH [19]. Cherry *et al*. [20] described that the peroxidase enzyme from *Coprinus cinerinus* had to be mutated broadly to allow this enzyme to be used as a detergent additive at pH 10.5. In the present study the peroxidase activity was found maximum at pH 8.0, indicating its uses in detergent additives. Antonopoulos et al. [21] reported the optimum pH of 9.9 with *Streptomyces* albus ATCC 3005.

Carbon and nitrogen sources

Medium optimization by one factor at time method involves changing the independent variable while fixing the others at certain level. Selections of appropriate carbon and nitrogen sources are crucial for the development efficient and economic process. Hence these two parameters were selected in the present work. Carbon and nitrogen sources supported the maximum peroxidase activity of the isolate *Bacillus* sp. Eight different carbon sources such as glucose, starch, carboxy methyl cellulose, fructose, mannitol, maltose and sucrose were tested at 1.0% for the production of peroxidase. Among the carbon sources, mannitol supported a maximum peroxidase activity of 38.2 U/mL at 18 h (Fig. 3). Further, different concentration of mannitol was also tested and found a maximum peroxidase activity of 40.6 U/mL in 1.5 % concentration compared to the rest of concentration (Fig. 4). Different organic and inorganic nitrogen sources such as peptone, beef extract, yeast extract, NH_4Cl, $NaNO_3$, $(NH)_2HPO_4$, NH_4NO_3 were tested for the production of peroxidase. Among them, yeast extract supported a maximum peroxidase production of 43.5 U/mL at 18 h (Fig. 5). Among the different concentration of yeast extract tested, *Bacillus* sp. produced a maximum enzyme of 45.6 U/mL in 1.5 % concentration at 18h compared to the rest of concentration (Fig. 6). On the other hand, 0.3% of $NaNO_3$ supported the maximum enzyme production of 40.9 U/mL at 18 h (Fig. 7).

Master and Field, [22] have reported the ligninolytic enzymes are produced during the secondary metabolism under conditions of limited nitrogen. The production of peroxidases is a common feature among bacteria. In the present study, peroxidase activity of *Bacillus* sp. was produced in a liquid medium in which the main carbon and nitrogen sources were mannitol and yeast extract respectively. Maximum peroxidase activity of *Bacillus* sp. was obtained when the medium was amended with 1.5% (w/v) mannitol and 1.5 % (w/v) yeast extract supplied in the culture medium . Antonopoulos et al. [21] reported the maximal peroxidase activity from *Streptomyces albus* ATCC 3005 was at 0.9% (w/v) oat spelt xylan and 0.6 % (w/v) yeast extract was supplied in the culture medium.

Among the inorganic nitrogen sources NH_4Cl gave low yields of peroxidase activity while maximum peroxidase activity of 38.4 U/mL was obtained using $NaNO_3$ in the present bacterium *Bacillus* sp. Hence $NaNO_3$ was selected as the best inorganic nitrogen source for the bacterium have reported the production of peroxidase from *Bacillus* sp.

Amino acid

Ten different amino acid sources such as glutamine, glycine, aspartic acid, alanine, methionine, phenylalanine, leucine, proline, asparagine and tyrosine were tested for peroxidase production in *Bacillus* sp at the concentration of 0.2%. Among them asparagine supported a maximum peroxidase production of 50.7 U/mL at 18 h (Fig. 8). Several researchers have also reported that some aminoacids are involved to enhance various microbial enzyme production during the optimization process [24, 25].

Figure 1. Effects of pH on peroxidase production by *Bacillus* sp.

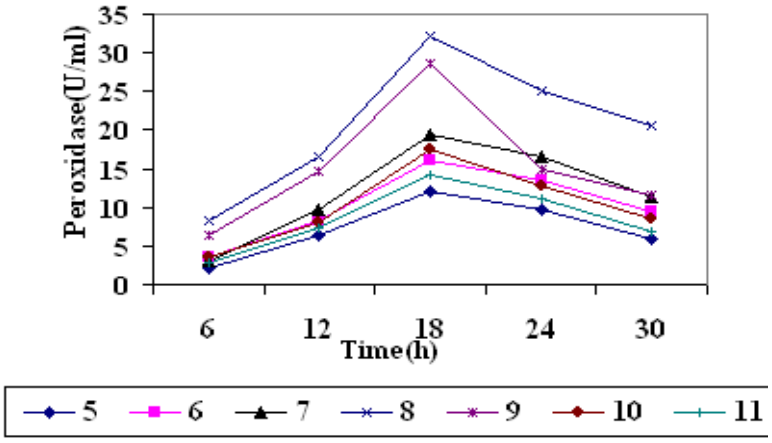


Figure 2. Effects of temperature (in degree celsius) on peroxidase production by *Bacillus* sp.

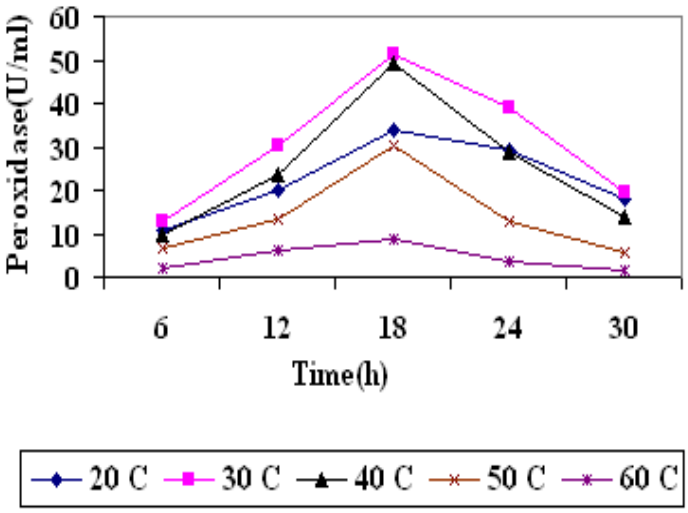


Figure 3. Effects of different carbon sources on peroxidase production by *Bacillus* sp.

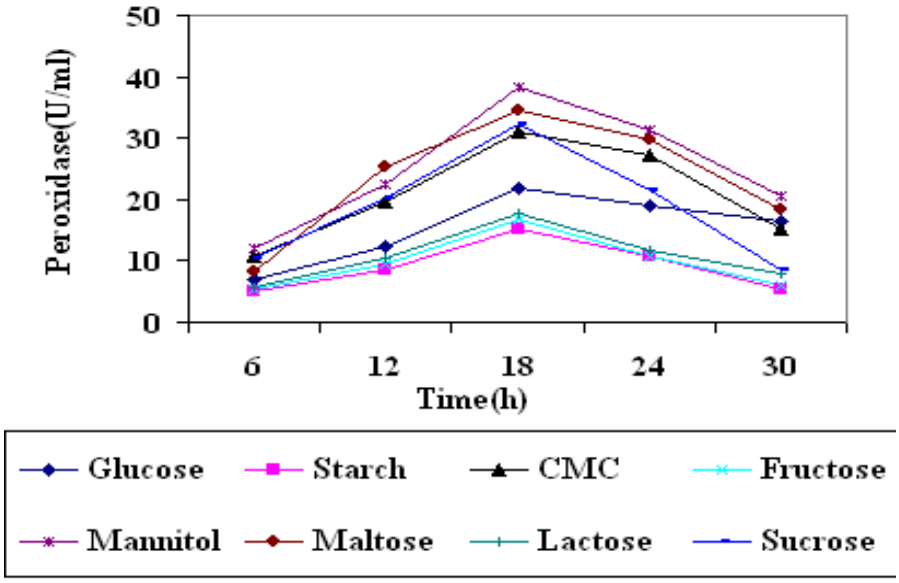


Figure 4. Effects of different concentrations (%) of mannitol on peroxidase production by *Bacillus* sp.

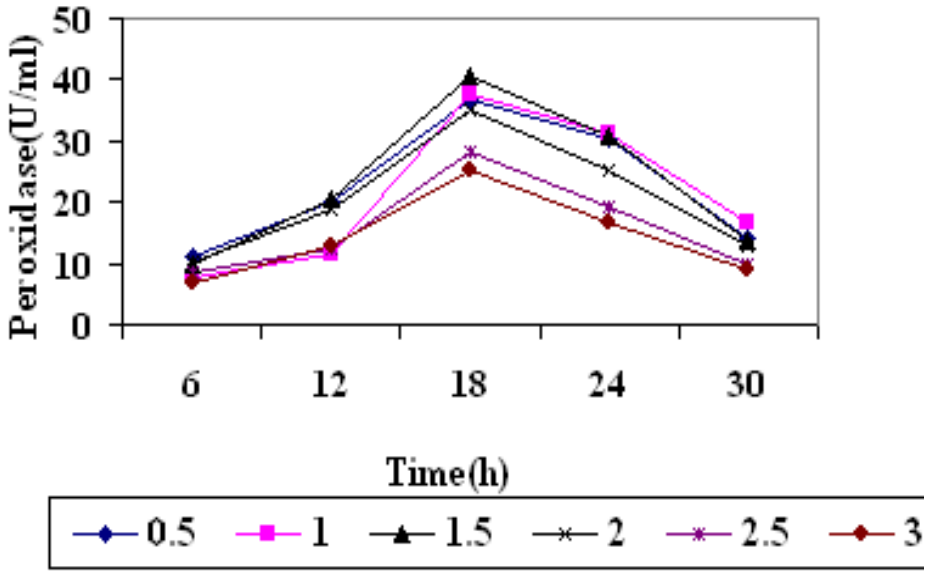


Figure 5. Effects of different nitrogen sources on peroxidase production by *Bacillus* sp.

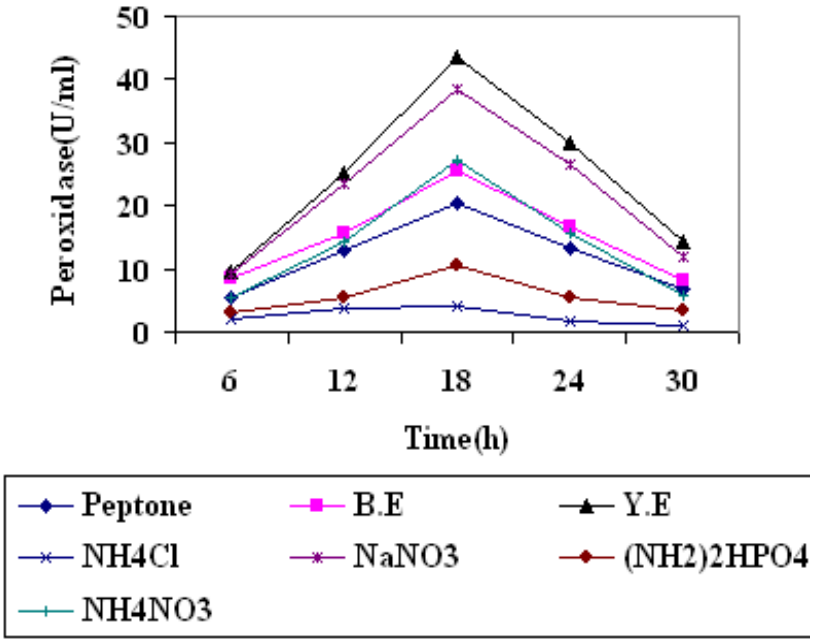


Figure 6. Effects of different concentrations (%) of yeast extract on peroxidase production by *Bacillus* sp.

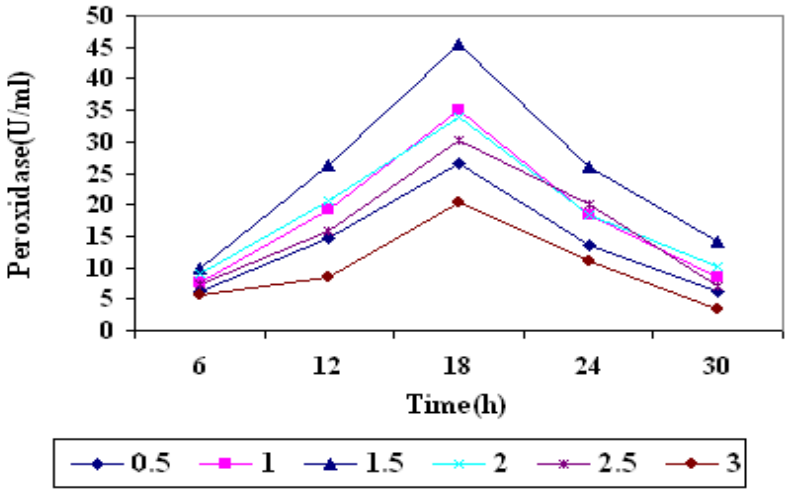


Figure 7. Effects of different concentrations (%) of $NaNO_3$ on peroxidase production by *Bacillus* sp.

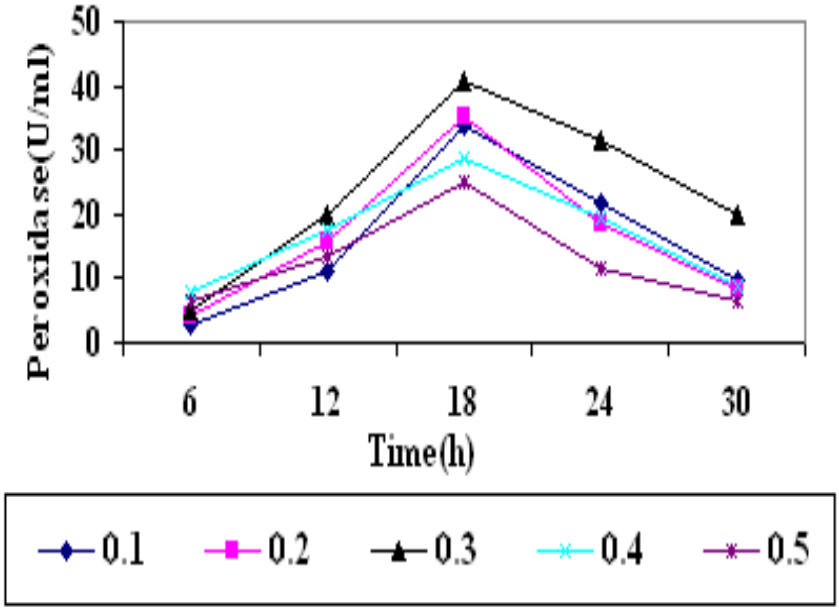
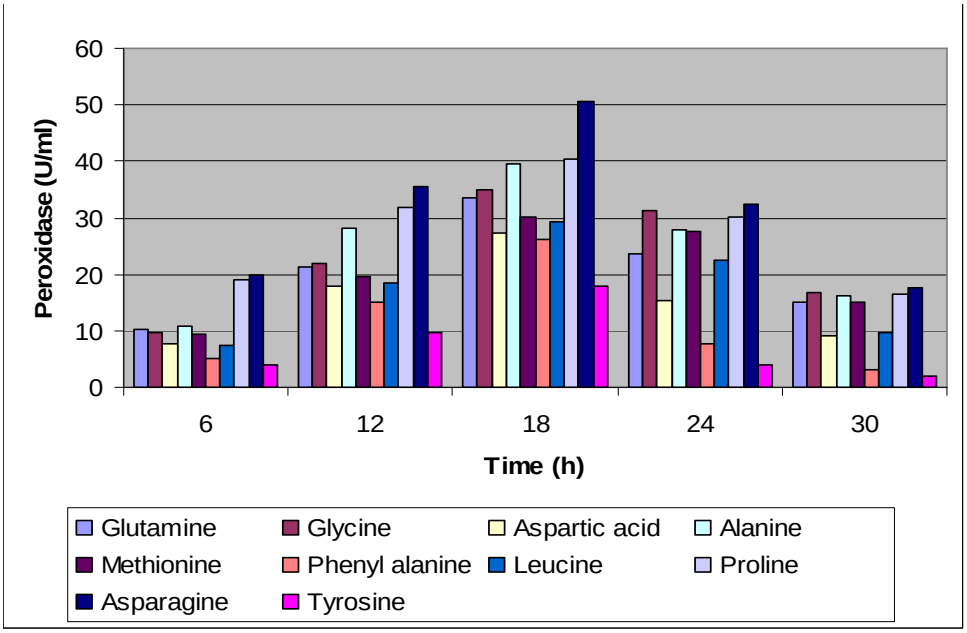


Figure 8. Effects of different amino acids on peroxidase production by *Bacillus* sp.



CONCLUSION

It is well understood from the present study that optimum pH, temperature, carbon and nitrogen sources are the limiting factors for the maximum production of peroxidase. The medium amended with 1.5% yeast extract, 0.3 % $NaNO_3$, 1.5% mannitol, 0.2% asparagine and pH 8, incubation temperature at 30°C for 18 h were supported maximum peroxidase production of 50.7 U/mL. It was concluded that the peroxidase obtained from *Bacillus* sp. suggesting its role in various industrial applications.

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