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Full Length Research Paper

Production of manganese peroxidase by white rot fungi from potato-processing wastewater: Role of amino acids on biosynthesis

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The production of manganese peroxidase (MnP) by white rot fungus strain L-25 was carried out using potato-processing wastewater and the effects of amino acids in the potato-processing wastewater was investigated. The MnP was efficiently produced from the wastewater by the addition of glucose and the maximum MnP activity linearly increased with an increase in the glucose concentration. The initial pH affected the cell growth and also the production rate of the MnP. The maximum activity and the production rate of the MnP using the potato-processing wastewater-based medium were higher (ca. 2.5-fold) than that of the basal medium. Moreover, amino acids in the wastewater had significant effects on the MnP production. L-Glutamic acid, L-aspartic acid and L-serine induced the MnP secretion, on the other hand, L-phenylalanine, L-tyrosine, L-leucine and L-lysine inhibited it. The addition of L-leucine and L-lysine caused growth inhibition, while, L-phenylalanine and L-tyrosine blocked the MnP biosynthetic pathway. Ammonium ion released from the L-phenylalanine by the L-phenylalanine ammonia-lyase participated in the repression of the MnP biosynthetic pathway of the strain L-25.

Key words: Manganese peroxidase, white rot fungus, potato-processing wastewater, amino acid, ammonium ion.

INTRODUCTION

Ligninolytic enzymes, such as manganese peroxidase (MnP) (EC 1.11.1.13), lignin peroxidase and laccase, are mainly secreted by white rot fungi and these degrade lignin from wood in the natural environment. These enzymes are able to degrade a variety of pollutants, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and synthetic dyes due to their low substrate-specificity (Levin et al., 2008). In recent years, therefore, the ligninolytic enzymes are expected to be used in bioprocesses, such as bioremediation, biopulping, biobleaching and bio-ethanol production (Rodríuez Couto et al., 2004). For these biotechnological applications, large amounts of enzymes must be supplied at a low-cost. Although the production of ligninolytic enzymes has been investigated from the viewpoint of culture medium and bioreactor design using white rot fungi, such as *Phanerochaete chrysosporium*, their productivities have not currently reached industrial levels except for the laccase.

As for the culture medium, food-processing wastes have been expected to be a low-cost source for the enzyme production. For example, the olive mill wastewater, mandarin peelings and kiwi fruit wastes were reported to be useful as culture media (Fenice et al., 2003; Rosales et al., 2005; Songulashvili et al., 2007). In the case of *Panus tigrinus* using olive-mill-wastewater as the basal medium, relatively high amounts of the MnP and the laccase were simultaneously produced with the decolorization of the colored wastewater and the degradation of

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the phenolic compounds (Fenice et al., 2003). These wastewater and wastes from the food-processing factory contain carbon and nitrogen sources that are essential for the growth of microorganisms and the production of useful materials, like enzymes. However, it was also reported that the type of wastes and their components affected the type and the amount of the ligninolytic enzy-mes production (Ruiz et al., 2002; Songulashvili et al., 2007). *Ganoderma lucidum* 447 produced only laccase when kiwi fruits, chicken feathers, corn bran or banana peels were used as the substitutes of glucose, whereas both the laccase and the MnP were produced when wheat bran, soy bran, mandarin peels or the residue of ethanol production from wheat grains were used (Songulashvili et al., 2007). Moreover, in the case of *Phanerochaete flavido-alba*, the two olive mill wastewater components (monomeric aromatic compounds and a brownish polymeric pigment) affected the ligninolytic enzyme production (Ruiz et al., 2002). The laccase was the sole ligninolytic enzyme detected in the cultures containing monomeric aromatic compounds, whereas both the laccase and the MnP were accumulated in cultures containing olive mill wastewater or polymeric pigments. Although several literatures have been reported concerning the ligninolytic enzyme production from wastes as already mentioned, there are few reports regarding the effect of the nitrogen sources contained in the wastes during ligninolytic enzymes production. Therefore, the analysis of the effect of nitrogen sources, such as amino acids, is a worthwhile topic, which will be the useful knowledge for the development of a low-cost culture medium, such as food processing wastes.

The potato-processing wastewater, which is generated in potato-starch production processes and causes environmental problems due to its large effluent volume and high nutrient contents, may be useful for the enzyme production as well as the olive-mill wastewater. In general, the white rot fungi, such as *P. chrysosporium*, secrete ligninolytic enzymes only after the exhaustion of all nitrogen or carbon sources in the culture medium. On the contrary, strain L-25 secretes MnP before the depletion of the nitrogen source using a nitrogen rich medium as was previously reported (Fujihara et al., 2008). In this study, therefore, the use of a potato-processing wastewater as a raw material for the MnP production was examined using the strain L-25. In addition, the effects of amino acids in the potato-processing wastewater on the MnP production were investigated.

MATERIALS AND METHODS

Chemicals

The potato-processing wastewater, which is generated during the potato starch production process, was supplied by Hitachi Plant Construction and Services, Japan. Its characteristics were as follows: pH 6.5, BOD 22000 mg/l, COD 13000 mg/l, total organic carbons 9500 mg/l and total nitrogen 1.72 g-N/L. Potato dextrose broth, Polypepton and potato dextrose agar were obtained from Becton, Dickinson and Company, USA, Nihon Pharmaceutical Co. Ltd., Japan and Nissui Pharmaceutical Co. Ltd., Japan, respectively. All other chemicals were of analytical grade.

Fungal strain

The white rot fungus strain L-25 was used throughout this study. It was grown on potato dextrose agar slants at 30°C for 5 - 6 d and then stored at 4°C as described by Kariminiaae-Hamedaani et al. (2007). This strain was found to belong to the Polyporales order based on an 18S rDNA analysis, but further identification could not be carried out.

Culture conditions

One mycelial piece of the strain L-25 was placed at the center of a potato dextrose agar plate and incubated at 30°C. After 5 d of incubation, one mycelial plug (1 cm diameter) was cut off from the edge of the mycelial mat and added to a 500 ml baffled Erlenmeyer flask containing 100 ml of the culture medium. The culture was carried out on a rotary shaker at 150 rpm and 30°C. Unless otherwise specified, each experiment was carried out at least in duplicate and the results were averaged.

The potato-processing wastewater was used without dilution and supplemented with glucose and $MnSO_4 \cdot 5H_2O$ so that the composition of the wastewater became similar to the basal medium as no growth was observed without additives. Unless otherwise stated, the basal medium (pH 7.0) was the potato dextrose broth (20 g/l glucose and 4 g/l potato infusion) supplemented with 0.1 mM $MnSO_4 \cdot 5H_2O$. The strain L-25 produced a relatively high amount of MnP using the potato dextrose broth in the study of Fujihara et al., (2008), thus it was used as a reference. The initial pH of each media was adjusted with a 0.5 M NaOH or 0.5 M HCl after autoclaving.

To verify the effects of the amino acids, 7 kinds of amino acids (that is, L-aspartic acid, L-glutamic acid, L-leucine, L-lysine, L-phenylalanine, L-serine and L-tyrosine) were selected. These amino acids were dissolved in distilled water and sterilized by filtration, which in turn were separately added to the basal media at a 0.5 or 1.0 g/l final concentration after autoclaving. Inorganic nitrogen sources (0.396 g/l ammonium sulfate, 0.321 g/l ammonium chloride and 0.510 g/l sodium nitrate) were added to the basal media at a 0.085 g-N/L before autoclaving. The initial pH was adjusted to 7.0 with 0.5 M NaOH or 0.5 M HCl.

Analytical methods

The MnP activity was measured based on the oxidation of Mn^{2+} to Mn^{3+} using sodium malonate buffer (Wariishi et al., 1992). The reaction mixture (3 ml) contained 50 mM disodium malonate (pH 4.5), 1.0 mM $MnSO_4 \cdot 5H_2O$, 0.1 mM H_2O_2 and the medium supernatant (100 µl). The reaction was started by the addition of a H_2O_2 solution (100 µl) and the rate of Mn^{3+}-malonate complex formation was measured at 270 nm for 1 min (ε = 11,590 M^{-1} cm^{-1}). One unit of MnP was defined as the amount of enzyme required to oxidize 1 µmol of Mn^{2+} to Mn^{3+} per minute at 25°C. In this experimental range, any additives such as amino acids and nitrogen sources did not affect the MnP activity measurement.

The glucose concentration was determined using the Glucose CII Wako kit (Wako Pure Chemical Industries, Japan) and the extracellular protein concentration was measured by the Bradford method (Bradford, 1976). After cultivation, the mycelia were harvested by centrifugation at 9,100 × g for 10 min and washed twice with distilled water under the same conditions. The mycelial weight

Table 1. Medium components of a potato-processing wastewater-based medium.

Components	Trials					
	1	2	3	4	5	6
PDB [ml]	100	-	-	-	-	-
PPW [a] [ml]	-	100	100	100	100	100
Glucose [g/l]	-	20	20	20	**10**	**30**
$MnSO_4$ [mM]	0.1	0.1	0.1	0.1	0.1	0.1
pH	7.0	5.0	6.0	7.0	6.5	6.5
Cost [b] * [Yen/100 ml-medium]	83.5	4.70	4.70	4.70	2.35	7.05

[a] PPW =potato-processing wastewater; [b] = Cost was calculated based on the reagent price at laboratory scale.
* = JPY/USD exchange rate was about 90 in December 2009.

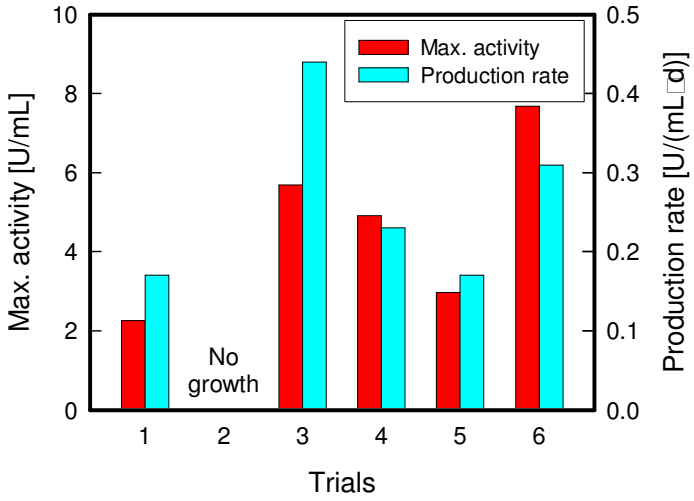

Figure 1. MnP production by the strain L-25 using the potato-processing wastewater medium.

was measured after drying at 100°C for 24 h. The amino acid concentration was measured with an L-8500 amino acid analyzer (Hitachi High-Technologies Corporation, Japan).

RESULTS AND DISCUSSION

MnP production by potato-processing wastewater-based medium

Since the glucose was scarcely contained in the potato-processing wastewater, the strain L-25 could not grow without additives. The glucose and manganese were added to the potato-processing wastewater so that the medium conditions became similar to the basal medium. As shown in Table 1, in trials 2 - 4, the concentrations of the additives (Mn^{2+} and glucose) were set to the same value as the basal medium and only the initial pH was changed in the range of 5 - 7. In trials 5 and 6, the glucose concentration was set in the range of 10 - 30 g/l. This experiment was not carried out in duplicate. Moreover, the cell concentration could not be accurately determined because the consumptive solids were included in the potato-processing wastewater. Comparisons of the maximum MnP activity and the production rate are shown in Figure 1. The initial pH and glucose concentration showed a significant effect on the MnP production when the potato-processing wastewater was used as the medium. The initial pH affected the cell growth and the production rate. Although the strain L-25 did not grow at all at the initial pH of 5.0, the maximum MnP activity and the production rate were higher (ca. 2.5-fold, respectively) than that of the basal medium at the initial pH of 6.0. In addition, the glucose concentration affected the maximum MnP activity. The maximum MnP activity increased with an increase of glucose concentration and no substrate inhibition was observed in this concentration range (0 - 30 g/l). Based on a visual observation, the cell concentration also increased with an increase of glucose concentration. In the case of the potato-processing wastewater-based medium, the vigorous consumption of glucose began on day 8 and its consumption rate was higher than that of the basal medium (Figure 2). The MnP activity increased with a decrease of glucose concentration and reached a maximum on day 13. Since this rapid consumption of glucose has not been observed for the basal medium, some effective ingredients must be included in the potato-processing wastewater.

By using the potato-processing wastewater-based medium, the cost for the medium became about 1/10-1/20 of that for the basal medium at the reagent level as shown in Table 1 and the MnP production became approximately 2-3 fold higher. Thus, the potato-processing wastewater would be a suitable medium source for the MnP production by the strain L-25.

Amino acid analysis of potato-processing wastewater

In general, white rot fungi are capable of producing ligninolytic enzymes only when a nitrogen-limited medium was used (Podgornik et al., 2001; Rogalski et al., 2006; Singh and Chen, 2008). On the contrary, the strain L-25 was able to produce high amounts of MnP even in a

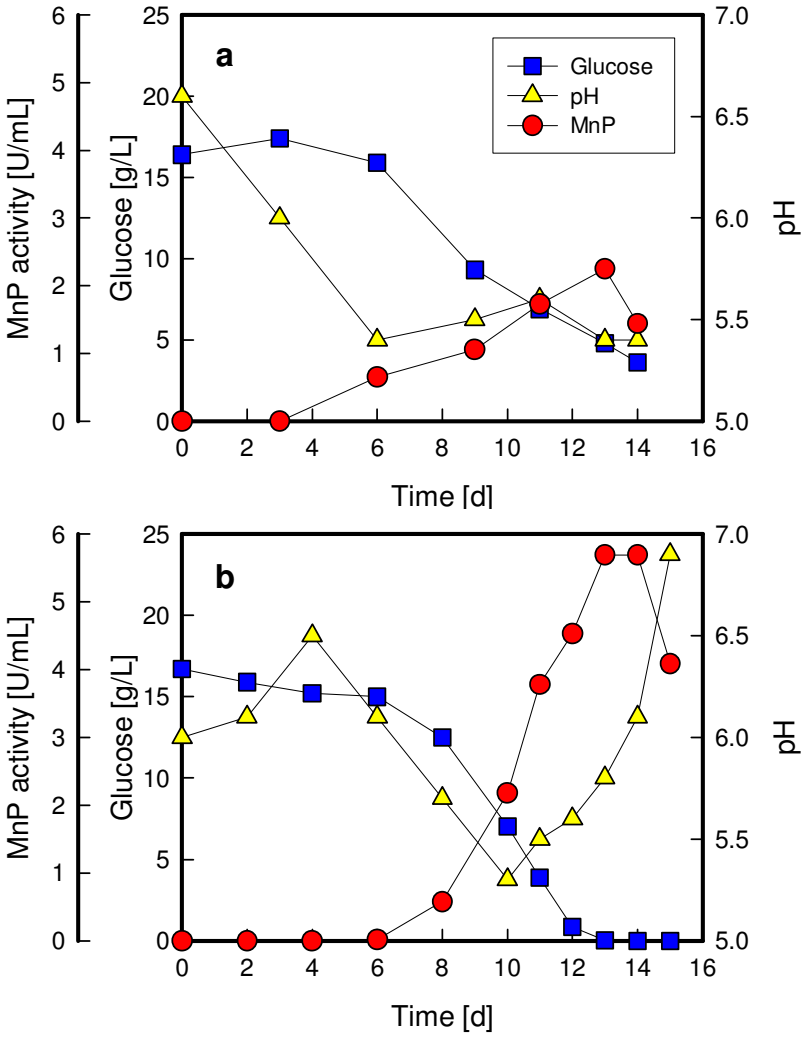

Figure 2. Time courses of MnP production using the basal medium (a, trial 1) and the potato-processing wastewater medium (b, trial 6).

nitrogen-rich medium as previously reported (Fujihara et al., 2008). This strain also showed a relatively high MnP activity in the potato-processing wastewater, which contained a high amount of nitrogen in the form of amino acids. Therefore, focus was on the amino acids as the nitrogen source. The result of the amino acid analysis is shown in Figure 3. The basal medium based on the potato dextrose broth contained a high amount of L-glutamic acid, while the contents of the other amino acids were not very high. On the other hand, L-phenylalanine and L-tyrosine were the most common amino acids in the potato-processing wastewater and the distribution of the amino acids in the potato-processing wastewater was different from that in the basal medium. No similarity was observed for the ingredients between the potato dextrose broth and the potato-processing wastewater, except that the contents of L-phenylalanine and L-serine in the potato dextrose broth were almost comparable with those in the potato-processing wastewater, respectively.

Effects of amino acids on MnP production

Based on the results of an amino acid analysis, the effects of the 7 amino acids on the MnP production were examined, that is, L-tyrosine, L-phenylalanine, L-serine, L-lysine and L-aspartic acid were selected as the main ingredient in the potato-processing wastewater and L-glutamic acid and L-leucine were selected as the main ingredients in the potato dextrose broth. Each amino acid was added to the basal medium at the 0.5 or 1.0 g/l level. Figure 4 shows the effects of the amino acids on the MnP production. L-Glutamic acid (main amino acid in the potato dextrose broth), L-aspartic acid and L-serine had a positive effect on the MnP production (Figures 4a - c). The maximum MnP activity linearly increased with the

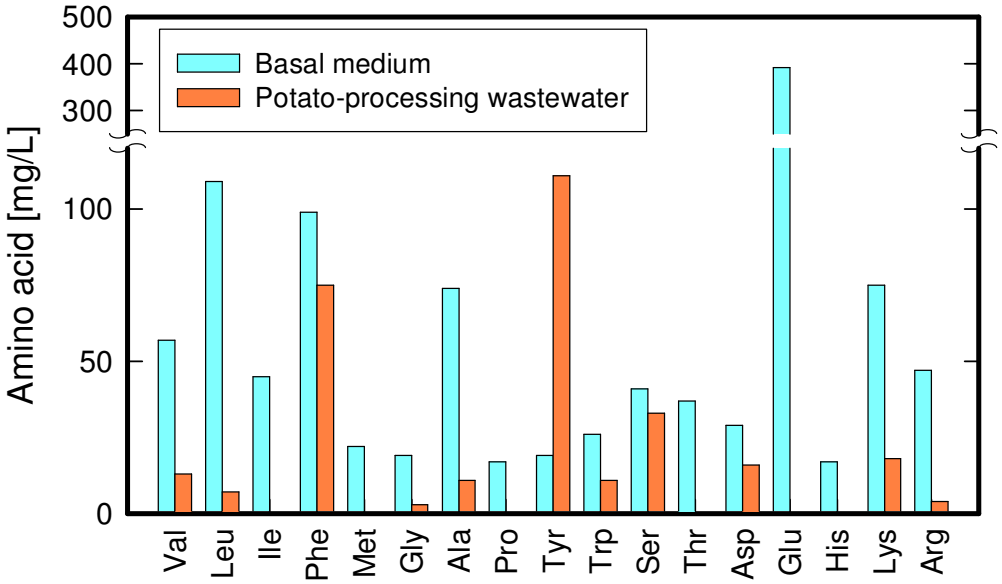

Figure 3. Comparison of amino acid composition between the basal medium and the potato-processing wastewater-based medium.

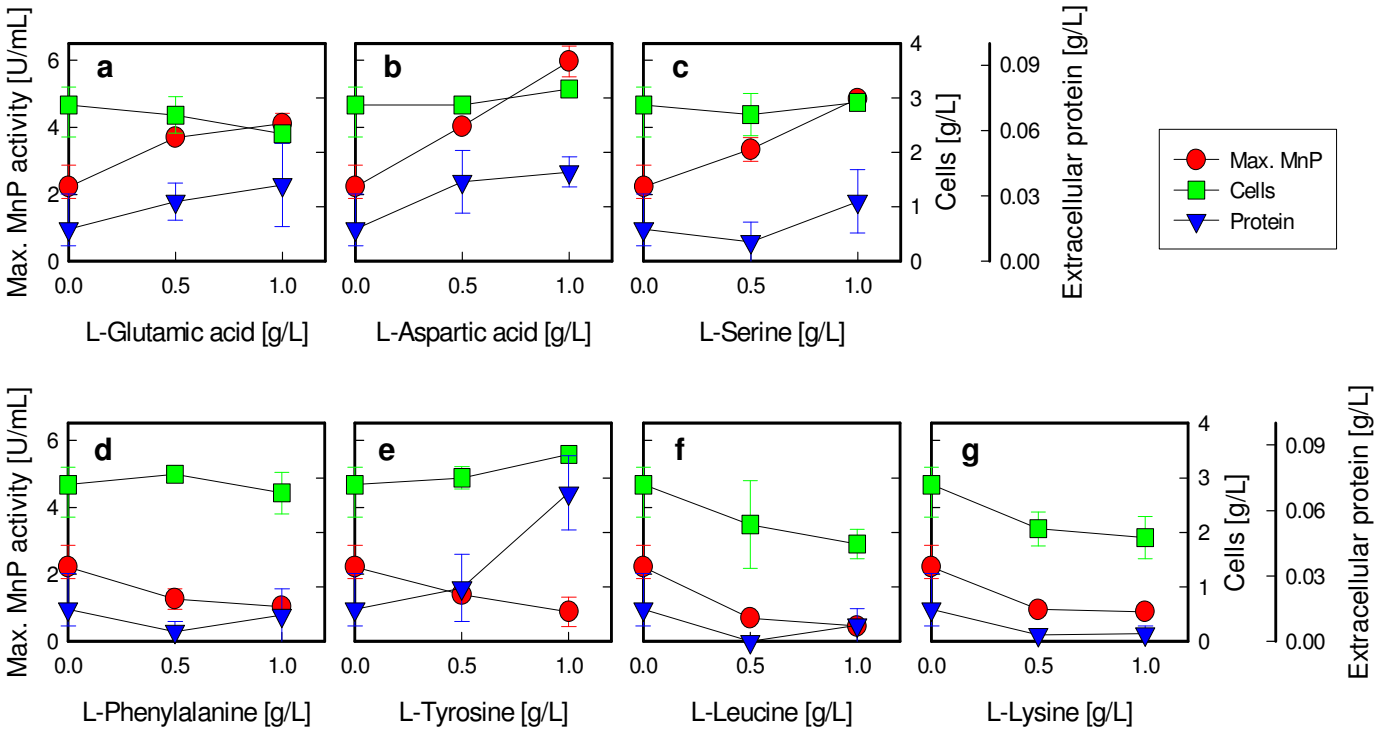

Figure 4. Effects of amino acids on MnP production by the strain L-25. The symbols show the average values and the error bars show the maximum and minimum values.

increase of amino acids concentrations in this experimental range. Their effects might be due to a change in the physico-chemical properties, such as membrane permeability, because their molecular structures are quite similar, that is, they have a short carbon chain with carboxyl or hydroxyl groups in their side chain. In contrast, L-phenylalanine, L-tyrosine, L-leucine and L-lysine had a negative effect on the MnP production (Figures 4 d - g). Because the addition of L-leucine and L-lysine decreased the cell concentration, the suppression of

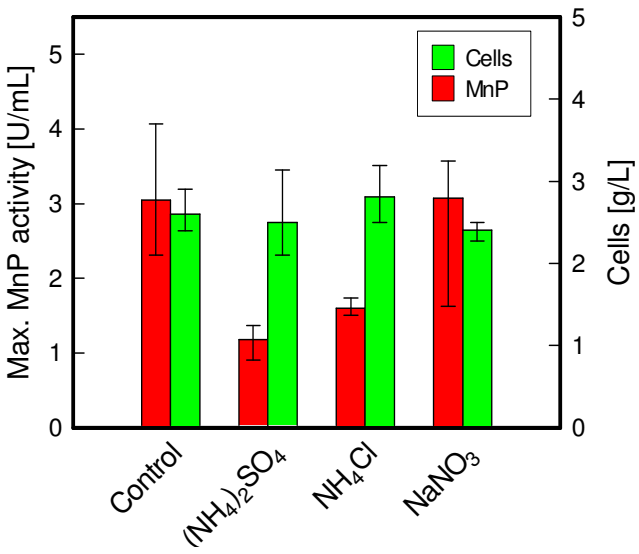

Figure 5. Effects of nitrogen sources on MnP production by the strain L-25. The symbols show the average values and the error bars show the maximum and minimum values.

the MnP production by these amino acids might be caused by growth inhibition. L-Phenylalanine did not affect the cell concentration and extracellular protein concentration, thus it must have block the MnP biosynthetic pathway. Only L-tyrosine increased the extracellular protein concentration without increasing the maximum MnP activity. Therefore, L-tyrosine could also have block the MnP biosynthesis and possibly induced the biosynthesis of other enzymes (or protein). Since L-phenylalanine and L-tyrosine, the main amino acids of the potato-processing wastewater, suppressed the MnP production, other active ingredients would be contained in the potato-processing wastewater.

It was reported that some amino acids inhibited the ligninolytic enzyme production by *P. chrysosporium* (Akamatsu and Shimada, 1996; Fenn and Kent Kirk, 1981), which belongs to a species similar to the strain L-25. L-Glutamic acid was the most effective amino acid regarding the suppression of the ligninolytic enzyme production by *P. chrysosporium*, which might be related to the ammonium ion (NH_4^+) released from glutamate by NAD-glutamate dehydrogenase (Fenn et al., 1981). In contrast, the MnP production by the strain L-25 was induced by L-glutamic acid in the present study. The participation of NAD-glutamate dehydrogenase in the suppression of the MnP production must be quite low in the case of the strain L-25. On the other hand, it was indicated that L-phenylalanine inhibited the MnP production due to the NH_4^+ released from the L-phenylalanine by L-phenylalanine ammonia-lyase (Hattori et al., 1999). In the case of the other white rot fungus strain, such as *Bjerkandera adusta* and *Trametes versicolor*, the L-phenylalanine ammonia-lyase was also induced by the addition of tyrosine (Xue et al., 2007). Since the L-phenylalanine ammonia-lyase recognizes not only phenylalanine but also tyrosine as the substrate and is also induced by tyrosine (MacDonald and D'Cunha, 2007), the inhibition of the MnP production by L-phenylalanine and L-tyrosine were presumably caused by the L-phenylalanine ammonia-lyase.

Effect of ammonium ion on MnP production

Since it was suggested that the L-phenylalanine ammonia-lyase participated in the repression of the MnP production by the strain L-25, the effect of the ammonium ion (NH_4^+), which will be released from L-phenylalanine by the catalysis of the L-phenylalanine ammonia-lyase, was investigated. Ammonium sulfate and ammonium chloride were used as the NH_4^+ source and sodium nitrate was also used for comparison. Each nitrogen source (0.085 g-N/L) was separately added to the basal media so that the nitrogen concentration became similar to that of 1.0 g/l L-phenylalanine. Comparisons of the maximum MnP activity and the cell concentration are shown in Figure 5. In the presence of the ammonium ions, the maximum MnP activity decreased to about half without a significant change in the cell concentration. This result was similar to that of L-phenylalanine. In contrast, there was no difference between the control and sodium nitrate (nitrate ion). This suggested that the ammonium ion released by the L-phenylalanine ammonia-lyase participated in the repression of the MnP biosynthetic pathway of the strain L-25.

Conclusion

The potato-processing wastewater is a promising medium source for the MnP production. The strain L-25 produced a relatively high amount of MnP (max. 7.7 U/ml) by using the potato-processing wastewater as the medium. The glucose concentration and the initial pH affected the maximum MnP activity and its production rate, respectively. Moreover, it was determined that amino acids in the potato-processing wastewater had a significant effect on the MnP production. L-Glutamic acid, L-aspartic acid and L-serine had a positive effect on the MnP production. In contrast, L-phenylalanine, L-tyrosine, L-leucine and L-lysine had a negative effect. L-Leucine and L-lysine caused a growth inhibition and L-phenylalanine and L-tyrosine blocked the MnP biosynthetic pathway. As shown in Figure 6, the inhibition by L-phenylalanine and L-tyrosine may be caused by the L-phenylalanine ammonia-lyase, because the addition of ammonium ion, which is released from L-phenylalanine, caused a similar inhibition. Since L-phenylalanine and L-tyrosine were the main amino acids in the potato-processing wastewater, other active ingredients to overcome these inhibitions would be present in the potato-processing wastewater. An investigation of this compound will be the next objective.

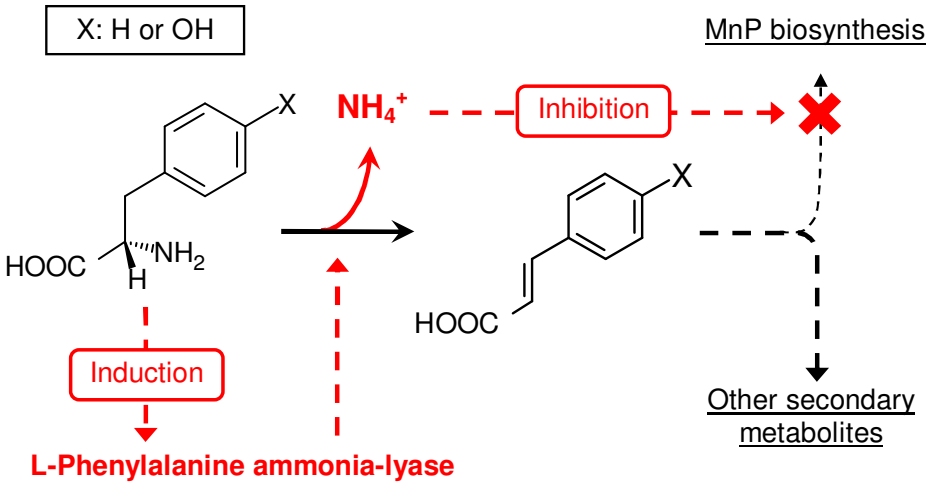

Figure 6. Inhibition mechanism of MnP production by L-phenylalanine (X = H) and L-tyrosine (X = OH).

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