Laccase Production with Submerged and Solid State Fermentation: Benefit and Cost Analysis

Batık ve Katı Faz Fermentasyonu ile Lakkaz Üretimi: Fayda ve Maliyet Analizi

Research Article

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ABSTRACT

The aim of this study is to investigate benefit and cost analysis of enzyme production with solid state and submerged fermentation techniques, biotechnological processes within production of value-added products. The approach of benefit and cost analysis is commonly used a way at deciding and investigating of the project. This analysis was referred two methodologies on production of laccase, a significant enzyme at environmental biotechnology. According to the result of the comparison, benefit/cost ratio in laccase activity obtained with solid state fermentation was calculated 1.76 and the ratio of laccase activity was 22.62 U ml^{-1}, whereas those of submerged fermentation was 2.42 and the activity was 29.08 U ml^{-1}. In particular, induced liquid culture obtained submerged fermentation is seen maximum benefit of production facilities and the minimum cost. This situation indicates laccase production with this type fermentation for a company is more attractive.

Key Words
Benefit-cost analysis, environmental enzyme, laccase, solid state fermentation, submerged fermentation.

ÖZET

Bu çalışmanın amacı, katma değer ürünlerin üretimlerine sahip biyoteknolojik süreçlerden katı faz ve batık fermentasyon teknikleri ile enzim üretiminin, fayda maliyet analizini değerlendirmektir. Fayda maliyet analiz yaklaşımı projenin karar verilmesinde ve değerlendirilmesinde genelde kullanılan bir yoldur. Bu analiz, çevresel biyoteknolojide önemli bir enzim olan lakkazın üretiminde kullanılan iki yöntemle ilgilidir. Karşılaştırma sonucuna göre, katı faz fermentasyonla elde edilen lakkaz aktivitesinde fayda/maliyet oranı 1.76 ve aktivite değeri 22.62 U ml^{-1} olurken batık fermentasyonla elde edilen lakkaz aktivitesi içinse bu değer 2.42 ve aktivite değeri 29.08 U ml^{-1}'dir. Özellikle batık fermentasyonla elde edilen indüklenmiş sıvı kültürü, üretim faaliyetinin maksimum fayda sağladığı ve minimum maliyet getirdiği görülmektedir. Bu durum bir şirket için bu tip fermentasyonla lakkaz üretiminin daha cazip olduğunu göstermektedir.

Anahtar Kelimeler
Fayda-maliyet analizi, çevresel enzim, lakkaz, katı faz fermentasyon, batık fermentasyon.

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INTRODUCTION

Solid state and submerged fermentation techniques are common and conventional biotechnology processes in view of production of value-added products such as enzyme, biopharmaceuticals, organic acid, biosurfactant, vitamin, flavoring compounds, biofuel, biopesticides etc. [1]. Solid-state fermentation (SSF) is described as the fermentation in absence or near absence of free water. SSF is known from ancient times (approximately 2600 b.c.) [2]. Submerged fermentation (SmF), more strongly developed from the 1940s onwards because of the necessity to produce antibiotics on a large scale has been characterized as fermentation in the presence of excess water. Almost all the large-scale enzyme producing facilities have been used the established approach of SmF owing to better monitoring and ease of handling [3].

Advantages of SSF over SmF are higher yields in a shorter time period, better oxygen circulation, resembling the natural habitat for filamentous fungi, lesser effort in downstream processing, performing better at wild-type strains of microorganisms than genetically modified ones, therefore, reducing energy and cost requirements. Lacking of catabolic repression brings about the better biomass growth in SSF process [4]. Viniegra-Gonz´alez studied strategies for the selection of mold strains to produce enzymes on solid conditions and found that higher enzyme titers than SmF, when comparing the same strain and fermentation broth [5]. Similar results were obtained from other studies [6,7].

Disadvantages of SSF over SmF are difficulty in scale-up and control of process parameters (pH, heat, nutritional requirements, etc.), lesser knowledge of this process, higher impurity product, thereby, increasing recovery product cost [8-11,2]. Thereby, SSF will never replace the established processes for the production of enzymes and metabolites by bacteria or yeasts that have for decades been successfully cultivated and optimized in SmF conditions [12]. Ramamurthy and Kothari observed that surface cultivation led to protease of low specific activity when compared to SmF and production cost was also higher [13]. Ramírez et al. found that laccase production by *Pleurotus ostreatus* grown on wheat bran and vinasse in SSF was twice as high (20 U l^{-1}) than those cultures grown in SmF [14]. According to the study of Téllez-Téllez et al. cultures grown in SmF produced laccase at 13.000 U l^{-1} [15]. However, cultures grown in SSF had a much lower laccase activity of 2.430 U l^{-1}. These results show that *P. ostreatus* performs much better in SmF than in SSF. Téllez-Jurado et al. liquid culture showed higher growth and greater production of laccase in SmF than in SSF [16]. To explain the molecular causes underlying for the different activities of enzymes in SSF and SmF, many studies have been performed. Oda et al. reported that the regulations at transcriptional level and posttranscriptional level are important at clarifying the formation of specific enzymes [17].

Most enzyme manufacturers have produced enzymes by these techniques. Enzyme production is a growing market of biotechnology with increasing number of patents and research reports [18]. Especially, ligninolytic enzymes such as laccase, manganese dependent peroxidase, and lignin peroxidase obtained with SSF or SmF offer potential advantages in bioremediation, detoxification and degradation of hazardous and toxic compounds to environment [1]. Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidases widely distributed among plants, insects and fungi. These enzymes catalyze the one electron oxidation of a broad variety of organic and inorganic substrates [19]. There is an increasing demand for laccase in the market for various applications such as biopulping [20], biobleaching [21-23], denim bleaching]24], organic synthesis [25], decolorization [26,28], dechlorination of xenobiotic compounds [29-31], bioremediation [32-35], plantfibre modification, ethanol production, wine stabilization, baking [36], cosmetic and dermatological preparations [37], biofuel cells *etc*. [38]. Laccase have offered a convenient and simple alternative way to supplying peroxidases with H_2O_2, owing to the fact that laccases are available on an economically feasible scale such as Suberase®, Lignozym® process, DeniLite® and ZyLite®.

Recently, there has been a rising development at the utilization of organic wastes such as residues from the agricultural, forestry and food industries to produce ligninolytic enzymes by SSF and SmF

technique [39]. The use of such wastes not only provides cheap substrates acting as inducers of the ligninolytic enzymes because of containing lignin or/and cellulose and hemicellulose, but also helps to solve environmental problems, which are otherwise caused by their disposal. Wheat bran, an agricultural waste, has extensively been investigated as a substrate in the laccase production with SSF and SmF methods [40,41].

Some researchers carried out a comparative economic analysis of SSF and SmF processes for the production of lipases by *Penicillium restrictum*. They found that for a plant producing 100 m^3 lipase concentrate per year, the process based on SmF needed a total capital investment 78% higher than the one based on SSF and its product had a unitary cost 68% higher than the product market price [42]. These results showed the great advantage of the SSF due to its low cost.

Benefit and cost analysis (BCA) is assessment of the economic efficiency criterion offering one method to aid decision-makers in this context. The approach of BCA analysis is frequently used a method at selecting and investigating of the project. The economy theory is based on benefits from a goods or service in addition to expense analysis for providing the goods or service.

Principally, BCA means that the sum of benefits or incomes during operation period of the project divides the sum of cost including the first investment amount throughout the project [43-45]. At this time, determination of benefits and cost is a main problem. Another problem is truly and elaborately measurement of benefits and cost [46]. However, moving to the present, thereby, the reduction of the identified benefits and costs is necessary by taking into account the current interest rate and emergence time. The resulting benefit-cost ratio depending on the amount of cash flow takes values from zero to infinite [44,47]. To sum up,

$$0 \leq B/C \geq \infty,$$

B/C =1 means that the sum of the reduced benefits to the present time is equal to the sum of the reduced costs. When total benefit increases, B/C ratio raises and the value is higher than 1 [48]. On the other hand, total cost enhances, B/C ratio declines and the value is lower than 1. The mentioned B/C ratio used to selection and evaluation of the project is accepted when the value is higher than 1 [47-49]. In this way, the rationale assessment of methods or technologies used in the production process of a facility or system can be made taking into consideration B/C ratio. Given the change and development in technology, benefit-cost analysis including to the technological processes will provide utility maximization.

In this study, benefit and cost analysis of enhanced laccase production solid state and SmF techniques were employed and B/C rates were investigated in respect of higher benefit and lower cost. When the possibilities of usability of laccase enzyme produced and improved technological processes are considered, examining of the most profitable manufacturing method and the option of investment for laccase production prior to application is a requirement. Benefit and cost analysis was done with refer to two separate studies, thesis studied by Gedikli and Kutlu on production of laccase under submerged and solid conditions, respectively [50,51]. The results of laccase production obtained by the studies were compared due to their benefits and costs.

MATERIAL AND METHODS

Microorganism and culture conditions

Trametes versicolor ATCC200801 was maintained on agar slants using a malt extract-agar (Fluka) medium. The fungus was subcultured in malt extract-broth (Merck) and incubated at 30°C for 4 days. Solid-state fermentation was performed using wheat bran as the solid substrate. Five grams of substrate taken in Erlen Meyer flasks was moistened with distilled water (15 ml). The contents were autoclaved at 120°C for 15 min. After subcultured mycelia were taken from an actively growing fungus, they were homogenized and inoculated 4 ml per Erlen Meyer including solid media. The Erlen Meyer flasks were incubated 30 °C statically for 15 days [50].

SmF was conducted in a flask with 100 ml medium containing 3 g of wheat bran and potato

dextrose broth and inoculated with a homogenized of mycelia (4 ml), and cultivated on a rotary shaker of 150 rev min^{-1} for 12 days [51].

Preparation of crude laccase

For obtaining crude laccase under solid state conditions, at the end of fifteen days, 40 ml of distilled water was added to medium and agitated in shaking incubator at the rate of 200 $revmin^{-1}$ (EdmundBühler-Labortechnik-Materialtechnik Johanna Otto GmbH). The contents were filtered through Whatman filter paper No. 1. The filtrate was centrifuged at 5000 rev min^{-1} for 15 minutes. These culture filtrates were used to determine enzyme activities.

For obtaining crude laccase under submerged conditions, at the end of twelve days, the contents were filtered through Whatman filter paper No. 1. These culture filtrates were used to determine enzyme activities.

Assay of enzyme activity

Laccase activity was assayed according to the protocol described and modified by Coll et al. [52]. To determinate enzyme activity, 0.1 ml of (culture filtrate) enzyme source was added to 4.9 ml of 0.1 M sodium acetate buffer (pH 4.5) and 1 mM guaiacol as substrate. The reaction mixture prepared was incubated at 37°C for 15 minutes. Enzyme activity in the tubes was measured by reading optical density in the UV-Visible spectrophotometer adjusted to 465 nm wavelength. (Schimadzu UV-2550). 1 U of enzyme activity was defined as the amount of enzyme that elicited an increase in A_{465} of 0.1 absorbance unit per minute. Incubations with denatured laccase served as a control. Enzyme measurements were carried out in triplicates and the average values were presented.

Benefit and cost analysis

For laccase production with SmF; wheat bran, potato dextrose broth and distilled water as research materials were used while wheat bran and distilled water were used for SSF. *Trametes versicolor* is the live material used for both methods. In this study, benefit-cost analysis of laccase activity acquired by two different methods was done. This analysis composed of three stages: determining of the benefits and costs (BC), the measurement of BC and the assessment of BC [44,45,48].

Chemical inputs per unit and electricity and water costs used in both methods were discussed as variable costs. The fixed costs which are the personnel and machinery costs have been considered in the context, for both methods, the same as those fixed costs were assumed to be excluded from evaluation.

The maximum activities of *T.versicolor* laccase provided with SmF and SSF methods were thought as benefit and directly evaluated measurable benefit. The total benefit and cost was calculated through the equations below (eqs 1 and 2) by analyzing the present value with one-year lifetime benefit and a 10% of discount rate.

$$B / C = \frac{\sum_{t=1}^{T} \frac{TB_t}{(1+i)^t}}{\sum_{t=0}^{T} \frac{TC_t}{(1+i)^t}}$$

(Equation 1) [44]

$$B / C = \frac{\sum TB_t^d}{\sum TC_t^d} \geq 1$$

(Equation 2) [44]

where t is the time, i; discount rate, B; benefits, TB_t, the total annual benefits, C; cost, TC_t, the total annual cost, TB_t^d; discounted total annual benefits, TC_t^d is the discounted total annual cost.

As a result of analysis, the production method with higher net benefit was determined and this was evaluated according to criteria of minimum cost-maximum benefit through detecting the B/C ratio values of higher than 1.

RESULTS AND DISCUSSION

In this research, benefit-cost analysis was done considering the obtained maximum laccase activity provided with SmF and SSF methods and the following findings were reached. Fig. 1 indicates induced with wheat bran laccase activity at SSF for 15 days while Fig. 2 shows induced with wheat bran laccase activity at SmF for 21 days. For the application phase of both methods, cost units were given in Table 1. As seen in Table 1, fixed costs are common for both methods and their evaluation were excluded. The chemical inputs and electricity costs have been taken as variable costs and unit cost corresponds to the amount unit was calculated.

As shown in Table 1, the total unit costs (TC) equal to the unit amount are 11.989 € for submerged enzyme production, while 12.794 € is that of solid state enzyme production. It can be seen that the cost of enzyme production with SmF is lower.

In this work, the maximum laccase activities were evaluated the scope of measurable benefit and laccase activity (TB) obtained with SmF was 29.08 U ml^{-1} whereas, 22.62 U ml^{-1} was that of laccase activity (TB) obtained with SSF (Table 2).

As can be seen in Table 2, the laccase activity obtained from submerged production is higher than activity produced SSF. The total of benefit and cost obtained separately for both methods was evaluated through an annual benefit life and 10% of discount rate, has been moved to the net present value and individual benefit-cost rates for two methods were achieved (Table 3).

Compared to the benefit and cost determined in Table 3;

For submerged fermentation culture;

$$B/C = \frac{\sum TB_t^d}{\sum TC_t^d} \geq 1 \Rightarrow$$

$$B/C = \frac{\sum 26.44}{\sum 10.899} = 2.42$$

2,42 ≥ 1

Table 1. The matrix of cost input for enzyme production with submerged and solid state fermentation.

Cost expenses	Production of induced enzyme with SmF		Production of induced enzyme with SSF	
	Amount per unit	Cost per unit [(C)(€)*]**	Amount per unit	Cost per unit [(C)(€)*]**
Constant cost				
Personnel cost		-		-
Machinery-instrument cost		-		-
Changeable cost				
Chemical inputs				
Wheat bran	15.0 (g/500 ml)	0.022	62.5 (g/500 ml)	0.093
Potato dextrose broth	12.0 (g/500 ml)	1.186	-	-
Distilled water	1500 ml	1.691	1187.5 ml	1.338
Other costs				
Electricity cost	1 KW	9.090	1.25 KW	11.363
Total cost (TC)		11.989		12.794

* Corresponding amount in the calculation of unit costs includes 18% value-added tax.

**30/09/2010 Calculation of unit costs in the Republic of Turkey Inflation Central Bank rate -adjusted exchange rate is used. 1 € was calculated as 1.98 TL.

For solid state fermentation culture;

$$B/C = \frac{\sum TB_t^d}{\sum TC_t^d} \geq 1 \Rightarrow$$

$$B/C = \frac{\sum 20\ .57}{\sum 11\ .630} = 1\ .76$$

1,76 ≥1

With the obtained data, the B/C ratio is greater than 1 for induced liquid culture method. However the B/C ratio is seen to be less than the other the production method for solid phase culture method. According to the criteria of low cost and high-benefit, laccase enzyme produced in submerged culture medium is more suitable in terms of application and results.

In this case, the production of induced submerged culture conditions should be preferred in practice because of utility maximization and cost minimization.

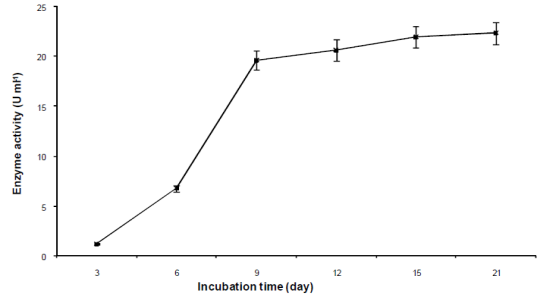

Figure 1. Induced with wheat bran laccase activity at SSF (Working conditions: incubation temperature, 30 ºC; inducer amount: 5 g/15 ml water)

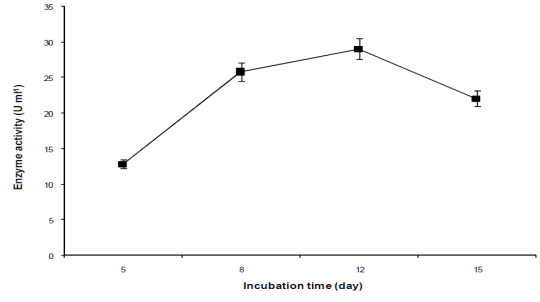

Figure 2. Induced with wheat bran laccase activity at SmF (Working conditions: Agitation rate, 150 r.p.m.; incubation temperature, 30 ºC, inducer amount: 3 g/100 ml medium)

CONCLUSIONS

The main hindrance at marketable application of laccase is being expensive of enzyme production.

Table 2. Matrix of measurable benefit unit for enzyme production with submerged and solid state fermentation

The methods used for enzyme production	Studied microorganism	Total benefit (B) (U ml^{-1})
SmF	T. versicolor	29.08
SSF	T. versicolor	22.62

Table 3. Benefit and cost ratios for each of two methods (i = %10)

Benefit time	SmF				SSF			
	TB_t	TC_t	TB_t^d	TC_t^d	TB_t	TC_t	TB_t^d	TC_t^d
0								
1	29.08	11.989	26.44	10.899	22.62	12.794	20.57	11.630
Total			26.44	10.899			20.57	11.630

TB_t =Total benefit, TC_t =Total cost, TB_t^d = Discounted total benefit, TC_t^d = Discounted total cost

Some attempts have been made over the last years to solve these problems and it is estimated that laccases will be able to compete with other chemical processes. Thereby, efforts have to be made in order to achieve cheap overproduction of this biocatalyst by using agro-industrial residues as substrate and their modification by protein engineering to achieve more active enzymes. When it was evaluated in terms of these research results, especially induced liquid culture is seen maximum benefit of production facilities and the minimum cost. In this case, laccase production with SmF for manufacturer is more attractive. Considering the possibilities of using the laccase, this enzyme production can be evaluated as the private or public investment project in respect of application technologies. Under the circumstances, determination of the benefits and costs to obtain from the project in question for the life of the project will be important. In the present case, proposed production methods at the stage of researching production possibility will offer a low cost and a high benefit for the practitioner, therefore, it will contribute to find common usage and the increase in production. Increased production and expansion of production method will accompany with the drop in prices, in this manner, it will facilitate the availability of the product and service and increase the effectiveness of the method.

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