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Research Article

Green Fluorescent Protein as an analytical tool in Process Analytical Technology

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ABSTRACT

Process Analytical Technology (PAT) is an initiative of US FDA to ensure for a better quality product with minimum cost, minimum wastage and defects by a constant check on the quality attributes throughout a process. The PAT concept proved beneficial in maintaining the quality of many of the pharmaceutical and biotechnological process. Yet for all there had been much opposition on implementation of PAT. Fermentation being one of the prime processes linking to most of the pharmaceutical products proves to be an excellent stand on this issue if at all PAT is successful in its case. Of the many techniques suggested by PAT for analyzing the quality of the process Green Fluorescent Protein (GFP), a step ahead in process analysis. The review aims at supporting the PAT concept with the discussion made on the benefits of GFP in various fermentation processes.

Keywords: PAT; US FDA; quality; fermentation; GFP

INTRODUCTION

Process Analytical Technology (PAT), an initiative taken by the US FDA in the year 2002, aimed at accessing the quality of all the manufacturing process related to human and veterinary products. The FDA has described it as," a system for designing and controlling manufacturing through timely measurements (i.e. during processing) of critical quality and performance attributes for raw and in-process materials and also processes with the goal of ensuring final product quality". The basic idea of PAT stands in examining the quality attributes of a process at three basic levels: (i) raw materials required in the process (ii) intermediate product and (iii) the final product (Fig. 1). The advantage of PAT lies in its predictive studies carried at each level of the process to correct or stop the process the moment anything goes wrong. This benefit by preventing any further loss of capital, material, energy and time, invested in the process.

Fermentation is one of the most commonly used biotechnological processes, which depends on the microorganism for the quality of the final product obtained. Presently, estimates made in any fermentation process include physical and chemical parameters that are performed in situ. With the implementation of PAT, newer analytical techniques like Near Infrared (NIR) Spectroscopy and Flow Injection Analysis (FIA) have proven to give satisfactory results however; due to the sensitivity of IR radiation to the water in the medium, the results obtained may not have accuracy. In such a situation, fluorescent proteins like the Green Fluorescent Protein (GFP) turn out to be an effective analytical tool for the on-line or at-line fermentation process (Känsäkoski et. al, 2006). In the present review, we are going to discuss the role of GFP in measuring the Critical Process Parameter (CPP) in the fermentation process, thereby supporting the concept of Process Analytical Technology.

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Green Fluorescent Protein

Aequorea Victoria species of jellyfish was studied for two of the proteins namely aequorin and green fluorescent protein (GFP), of which the latter shows its application as an efficient analytical tool. Many of the marine species have similar kind of GFPs but it is originally referred to that obtained for the *A.victoria*.

GFP is 4-(p-hydroxybenzyldine)-imidazolidin-5-one. With the folding to the native conformation followed by nucleophilic attack, dehydration and finally oxidation to form chromophore (Fig. 2). This would then result in a polycyclic aromatic system showing better fluorescence (Tsien R.Y, 1998). A colour shift of the aequorin from the blue at 470 nm, to the green colour of the green fluorescent protein at 570 nm is seen when the aequorin reacts with Ca^{2+} (Morise H.et al, 1974) which is hydrophobic and is solvated in ethanol. Of the 238 amino acids present in this fluorescent proteins the tripeptide serine, tyrosine and glycine forms the chromophore. The 11β strands of the GFP renders resistance to change by denaturation by temperature and pH (Ibtesam A.et al, 2009). GFP is structurally rigid and the hydroxyl group in the chromophore is responsible for the ability of the protein to fluorescence.

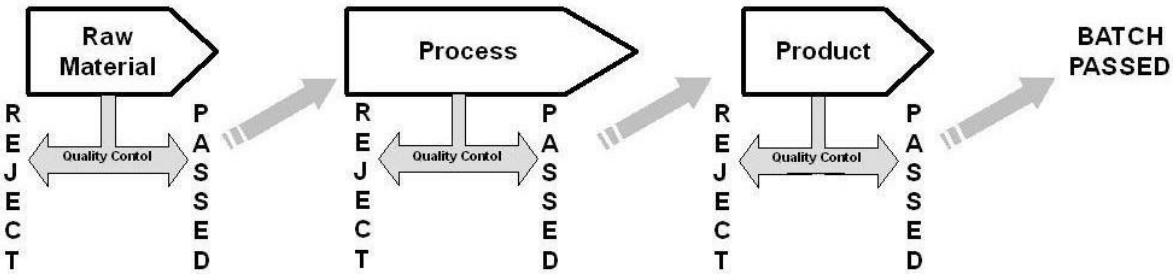

Figure 1: Outline for basic concept of Process Analytical Technology

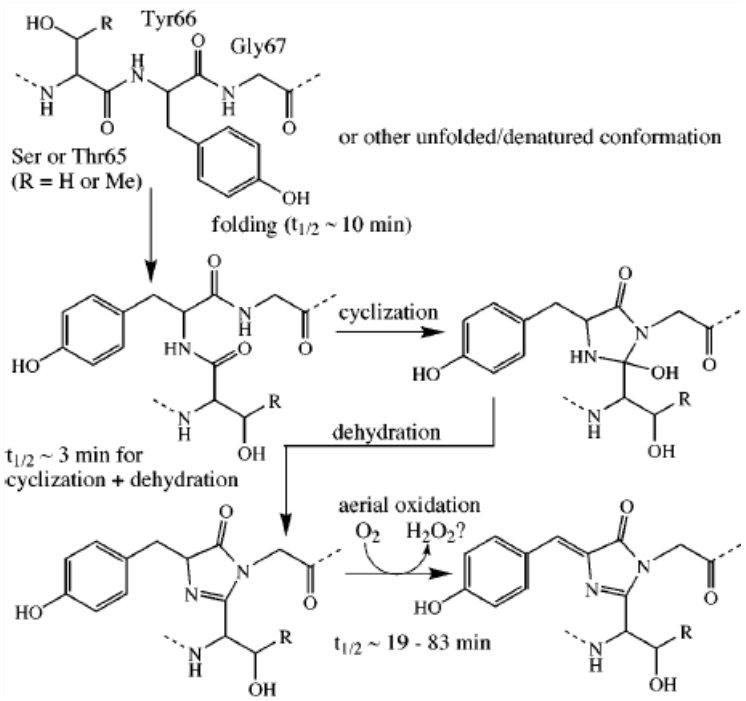

Figure 2: Formation of GFP chromophore (TsienR.Y, 1998)

The excitation of the protein from the singlet ground state to the singlet excited state occurs at 395 nm with a greater peak or at 475 nm with a smaller peak. Once the excitation reaches its maximum, the emission of radiation in the form of fluorescence is seen where in the energy is continuously lost till the fluorescent molecule reaches back to the singlet ground state. The emission peak is reached at around 509 nm.

GFP fluorescence is influenced by factors like the presence of reactive oxygen species, pH of the environment and the lack of oxygen. Amongst the various different classes of the GFP, the one with phenolate anion is most widely used. The wild type of the GFP, excites at 470 nm at alkaline pH due to the deprotonation of the 4-hydroxy group while in the acidic pH the protonation of the 4-hydroxy leads to excitation at 395 nm. Based on this pH sensitivity of the protein, the GFPs are chosen for the analysis of the organelle pH. GFPs are temperature sensitive and hence at 78°C, when denaturation is observed, around half of the fluorescence is lost. All these factors lead to quenching wherein the fluorescence is not detected by the spectrophotometer which in itself proves to be useful as an analytical tool.

Yeast in Fermentation

In fermentation the critical process is the conversion of the carbohydrate source into ethanol with the prime involvement of an enzyme. The successful conversion of the fermentation material to ethanol is possible only if the enzyme is maintained in a good condition till the entire process gets complete. *Saccharomyces cerevisae*, *Escherchia coli,* Lactococcus lactis, *Hansenula polymorpha* have been used widely as the fermenting enzyme for the GFP to express in them. This expression will aid in detecting the stress conditions, changes in temperature, oxygen and nutrient availability.

SDS-PAGE, RAPD, differential media, magnetic resonance are amongst the conventional methods used to determine the contamination of the yeast used for fermentation thus estimating the quality of the fermented matter. The protein present in the enzyme is targeted with a common purification tag and a fluorescent marker, like GFP, to measure the product concentration. GFP has an additional advantage of expressing in both prokaryotic and eukaryotic cells (Chalfie M. et al, 1994). GFP expressed by the pYGFP3 plasmid and transformed into Saccharomyces cerevisae was used to detect the contamination of the fermentation fed by

the spoiled yeast (Luiz H. G. et al,2000). With the help of an optic probe the expression of the fluorescent proteins was measured in a bioreactor during its on-line process (Randers L.et al., 1997).

Monitoring of the fluorescence

In the work done by Tobias Broger et al., the commercially used optical Aquasant probe was showed to quantitatively measure fluorescence of the expressed fluorescent reporter during the fermentation process. The probe is commercially used to measure the fluorescence by the turbidity measurement. The probe design suggested, involved a LED source that emitted the required excitation wavelength with the help of optical fibers and the emitted fluorescent wavelength was filtered and measured by the detector. Their comparative study made on the results obtained from the fluorescent reporter in on-line measurement and that of the conventional laboratory analytical methods showed that the fluorescent reporter measurement was a time saving, undelayed process with moderate expense giving quantitative measurements (Tobias B.et al, 2009).

FRET (Fluorescence or Förster Resonance Energy Transfer) as biosensor involves GFP with a modified GFP generally a BFP (Blue Fluorescent Protein) which give different colour other than green on emission. A spectral overlap is observed when the emission spectrum of one GFP (donor) turns to be the absorption spectrum for the other GFP (acceptor). In this way, any change in the physiological or biochemical signal can be estimated by FRET (Lakowicz J.R., 1983).

Other Applications

GFP, apart from its role as an analytical tool, is used for various other purpose. It has been used for genotoxicity monitoring in E.coli which expressed GFP, using a 2D-spectroscopic genotoxicity biosensing system (Bartolome A.J et al., 2003). As an application to FRET it has been used to monitor the interaction between a GFP fused protein and molecules like antibodies and proteins labeled with a fluorescent molecule. Intracellular monitoring of cAMP and Ca^{2+} for estimating the intracellular concentration has also been successful with FRET (Tavaré J.M. et al., 2001). GFP fused transgenic animal are being used as model for disease as well for other useful purpose.

CONCLUSION

PAT have shown its importance in many of the processes concerned with the pharmaceutical industry with no exception to the production of biological products,fermentation being of core importance in production of dextran, antibiotics like penicillin and many others. GFP alone can be used only in monitoring the activity of bacteria, yeast or enzymes involved in the fermentation process. Monitoring of the raw material and final product, however, needs further analytical procedures to ensure a product of good quality. PAT, although opposed by some, stands to be more effective in controlling the further damage or loss due to poor quality which is generally neglected. This not only prevents the loss of capital invested for the process on time but also reduces the risk of recalling of the product from the market.

PAT	Process Analytical Technology
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
NIR	Near Infrared Spectroscopy
FIA	Flow Injection Analysis
IR	Infrared Spectroscopy
CPP	Critical Process Parameter
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
RAPD	Random Amplified Polymorphic DNA
BFP	Blue Fluorescent Protein
FRET	Förster Resonance Energy Transfer

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