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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 product proves to be an excellent in 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) is a step ahead in process analysis. The review aims at supporting the PAT concept with the discussion made on the benefits of GFP as an analytical tool in PAT.

KEYWORDS: The PAT concept proved beneficial in maintaining the quality of many of the pharmaceutical and biotechnological process.

INTRODUCTION

Process Analytical Technology (PAT) has been defined by the United States Food and Drug Administration (FDA) as a mechanism to design, analyze, and control Pharmaceutical Manufacturing process through the measurement of Critical Process Parameters (CPP) which affect Critical Quality Attributes (CQA). [1] The concept actually aims at understanding the processes by defining their CPPs and accordingly monitoring them in a timely manner and thus being more efficient in testing while at the same time reducing over-processing, enhancing consistency and minimizing rejects.[2]

PAT involves defining the Critical Process Parameters (CPPs) of the equipment, used to make the product which affect the Critical Quality Attributes (CQAs) of the product and then controlling these CPPs within defined limits. This allows manufacturers to produce products with consistent quality and helps to reduce waste and overall costs.[1]

The basic idea of PAT stands in examining the quality attributes of a process at three basic levels.

- Raw material required in the process
- Intermediate product
- Final product

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.[2] Fermentation is one of the most commonly used biotechnological process 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 sensitivity of IR radiation to the water in the medium, the results obtained may not have accuracy. In such a situation, Fluorescent Protein like the Green Fluorescent Protein (GFP) turn out to be an effective tool for the on-line at-line fermentation process.[1]

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.

GFP is a protein composed of 238 amino acid residues (26.9KDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. GFP is 4-(p - hydroxy benzylidine)-

imidazolidin-5-one. With the folding to the native conformation followed by nucleophilic attack, dehydration and finally oxidation to form chromophore. This would then result in a polycyclic aromatic system showing better fluorescence.[3]

BASIC CONCEPT OF PAT

Process Analytical Technology (PAT), is an initiative taken by the US FDA in the year 2002, aimed at accessing the quality of all 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.[1]

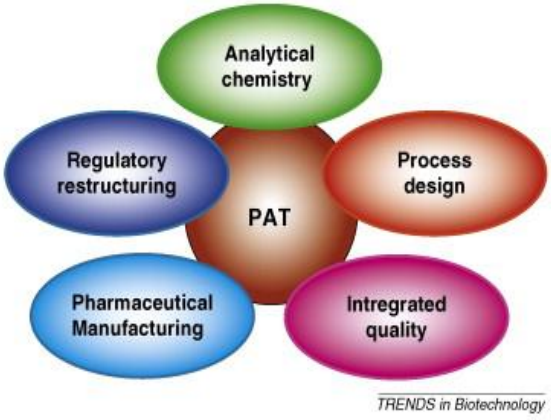

Fig 1: Major area covered by PAT

Basically, PAT involves a fundamental shift from testing the quality of the finished dry product, to building quality into products by testing at several intermediate steps. It specifically requires that quantifiable, casual and predictive relationship be established amongst raw materials, the manufacturing process, and final product quality. It is believed that PAT may not bring dramatic changes overnight, but years from now, it may be seen as an initiative that helped foster a period of innovation, efficiency and expansion for the biopharmaceutical industry. [2]

Process Analytical Technology Tools[2]

There are many current and new tools available that enable scientific, risk managed pharmaceutical development, manufacture, and quality assurance. These tools, when tested within a system can provide effective and efficient means for acquiring information to facilitate process understanding, develop risk mitigation strategies, achieve continous improvement, and share information and knowledge. In the PAT framework, these tools can be categorized as.

- ❖ Multivariate data acquisition and analysis tools
- ❖ Modern process analyzers or process analytical chemistry tools
- ❖ Process and end point monitoring and control tools
- ❖ Continous improvement and knowledge management tools

The desired goal of PAT framework is to design and develop processes that can consistently ensure a predefined quality at the end of the manufacturing process. Such procedures would be consistent with the basic tenet of quality by design and could reduce risk to quality and regulatory concerns while improving efficiency .

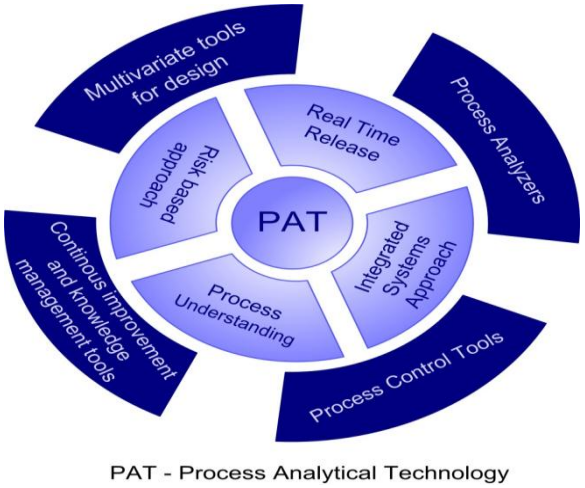

Fig 2: PAT Tools

Gains in quality, safety and/or efficiency will vary depending on the product and are likely to come from.

- Reducing production cycle time by using on-, in-, and /or at line measurements and controls.
- Preventing rejects, scrap, and re- processing.
- Considering the possibility of real time release.
- Increasing automation to improve operator safety and reduce human error.
- Facilitating continous processing to improve efficiency and manage variability.
  - Using small scale equipment and dedicated manufacturing facilities.
  - Improving energy and material use and increasing capacity.

Scope [2]

The potential benefits to industry include the following:

- ➢ Better understanding of processes.
- ➢ Batch to batch reproducibility.
- ➢ Fewer batch failures.
- ➢ Regulatory relief.
- ➢ Increased operating efficiency.
- ➢ Cycle time reduction.
- ➢ Close coupling of batch steps to produce semi continuous operations.

- The ability to use large scale processing equipment.
- Greater utilization of production equipment.
- Minimized storage space required for Work in Progress (WIP)
- Reduced risk of processing errors.
- Reduced risk of product contamination, by products and product modification.
- Minimized variability using on-line measurements.
- Reduce production cycling time.
- Prevent rejection of batches.
- Enable real time release.
- Increase automation.
- Improve energy and material use.
- Facilitate continous processing.

GREEN FLUORESCENT PROTEIN

Green Fluorescent Protein is a protein produced by a jellyfish *Aequorea victoria;* which produces glowing points of light around the margin of its umbrella. The light arises from yellow tissue masses that each consist of about 6000-7000 photogenic cells. These cells generate light by a process of bioluminescence, whose components include a calcium- activated photo protein (aequorin) that emit blue –green light and an accessory green fluorescent protein (GFP) which accepts energy from aequorin and re-emits it as green light. Many of the marine species have similar kind of GFPs but it is originally referred to that obtained from the A. victoria. [1]

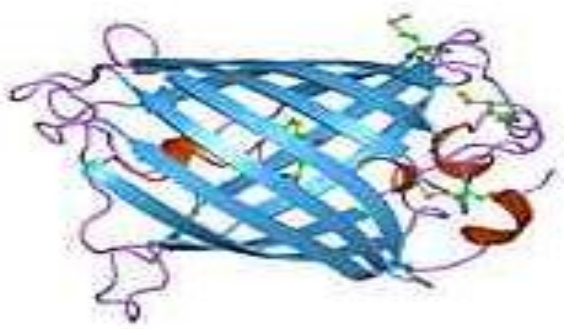

Fig 3: Structure of the Aequorea victoria green fluorescent protein

A color shift of the aequorin from the blue at 470nm, to the green color of the green fluorescent protein at 570nm is seen when the aequorin reacts with ca^{2+} which is hydrophobic and is solvated in ethanol. **[4]** Of the 238 amino acids present in this fluorescent protein the tripeptide serine, tyrosine and glycine forms the chromophore. The 11 beta strands of the GFP renders resistance to change by denaturation, by temperature and pH. **[5]** GFP is structurally rigid and the hydroxyl group in the chromophore is responsible for the ability of the protein to fluorescence.

The excitation of the protein from the singlet ground state to the singlet excited state occurs at 395nm with a greater peak; or at 475nm 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 509nm. **[1]**

The GFP - like proteins allow the monitoring in time and space of an ever increasing phenomena in living cells and organisms like gene – expression, protein localization and dynamics, protein –protein interactions, cell divisions, chromosome replication and organization, intracellular transport pathways, organelle inheritance and biogenesis. In addition, fluorescence from single GFP molecule has made it feasible to image at a spatial resolution higher than the diffraction limit. Furthermore, sensors that report pH values, calcium ion concentrations and other essential features of the interior of living cells have been engineered from GFP – like proteins.

STURUCTURE OF GFP

The crystal structure of GFP is an eleven-stranded β-barrel, threaded by an α-helix, running up along the axis of the cylinder. The chromophore is in the α-helix, very close to the centre of the can-like cylinder. A very large part of the primary structure of the protein is used to construct the β-barrel and the threading α-helix. The N-terminal residue and the C-terminal residues 230-238, approximately corresponding to the maximal numbers of residues that can be removed from the N-(2 residues) and C-terminal (6 residues) respectively of GFP at retained fluorescence, are disordered and therefore unresolved in this structural image.[6]

Fig 4: The tertiary structure of GFP, displaying its can-like shape with the α-helix, containing the chromophore, threading up through the can [7]

The polypeptide backbone folds into a previously unobserved motif: a beta- barrel of eleven strands, surrounding a central helix. Short distorted helical segments cap the barrel ends and help isolate the internal chromophore from solvent. An all-atom representation appears as a nearly perfect cylinder of about 25A° in

diameter and 40A° tall. Despite its monolithic appearance, GFP can be expressed as two separate segments that nevertheless combine within cells to yield a functional fluorescent protein. This discovery has led to useful applications, for example in the two-hybrid scheme, the individual GFP segments are fused to two other proteins and the resulting genes expressed in parallel. Appearance of visible fluorescence suggests that the two fusion partners tightly interact in vivo. The amino acid sequence of GFP can also be circularly permuted, resulting in new N- and C-termini, without loss of function. Inspection of the fold suggests that it can be divided into sequential segments, for example, an N-terminal portion (roughly 1–80) consisting of three beta strands plus the central helix, and a larger C-terminal portion (roughly 81–238) that consists of eight beta strands arranged in a typical 'Greek key'' motif. [8][9]

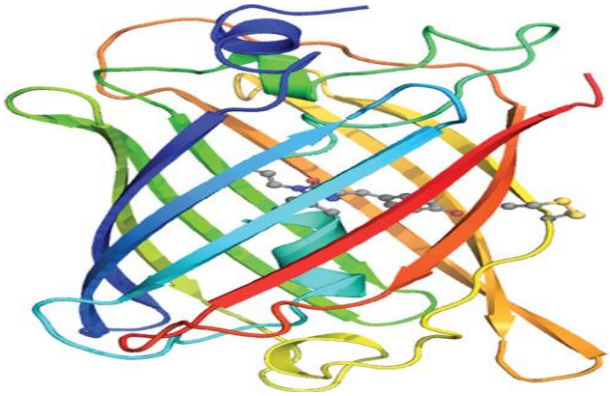

Fig5: Schematic diagram showing the backbone fold of GFP

FORMATION OF GFP CHROMOPHORE

A most interesting feature of GFP is that its function is based on a chromophore formed through a rarely observed autocatalytic post translational cyclization of a peptide from its own backbone structure. Initially it was believed that the autocatalytic GFP cyclization was unique, but recent research has indicated that a family of enzymes, including histidine ammonia lyase (HAL) and the closely related phenylalanine ammonia lyase (PAL), also contain post translational ring formations that occur auto catalytically through the attack of the protein backbone on itself. [10][11]

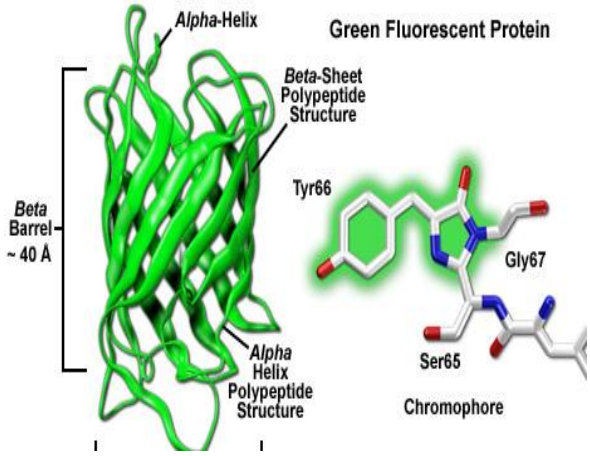

Fig6: GFP and its chromophore

The native green fluorescent protein (GFP), first so named by Morin and Hastings, from the jellyfish *Aequorea victoria* contains 238 amino acids. [12] Residues 65-67 (Ser-Tyr-Gly) in the GFP sequence spontaneously form the fluorescent chromophore p-hydroxybenzylideneimidazolinone.[7][13]

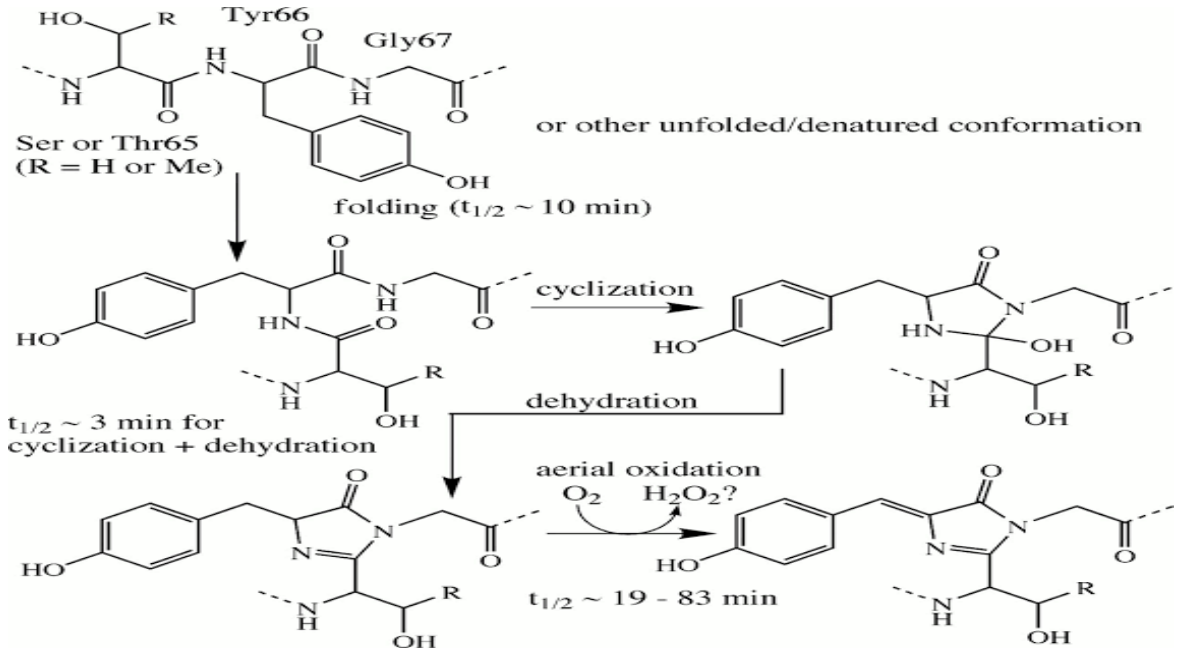

Fig7: Chemical reaction scheme accounting for the spontaneous formation of the GFP chromophore from Ser65-Tyr66-Gly67 motif in the native conformation of the protein in the presence of molecular oxygen. [3]

The tripeptide motif Ser65-Tyr66-Gly67- in the primary structure of unfolded or denatured GFP does not display any striking feature. But, as the GFP protein folds into its native conformation, these three amino acids are forced into a sharp turn, greatly favouring a nucleophilic attack of the amide of Gly67 on the carbonyl of Ser65, leading to imidazolinone formation by cyclization and dehydration. At this point, GFP does not fluoresce but, conditional on the presence of molecular oxygen, the α–β bond of residue 66 is subsequently dehydrogenated into conjugation with the imidazolinone which results in maturation of the GFP chromophore to its fluorescent form.[14][15]

FACTORS AFFECTING GFP FLUORESCENCE [1]

GFP fluorescence is influenced by factors like :

- presence of reactive oxygen species,
- pH of the environment and
- lack of oxygen.

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

ADVANTAGES OF GFP[16][17]

- The biggest advantage of GFP is that it is heritable, since it is able to be transformed with the use of DNA encoding GFP.
- Additionally, visualizing GFP is non invasive; it can be detected by simply shining a light.
- Furthermore, GFP is a relatively small and inert molecule, that doesn't seem to interfere with any biological process of interest.
- Moreover, if used with a monomer it is able to diffuse readily throughout cells.
- Expressed fusion proteins are generally not toxic to cells.
- Importantly, detection does not require fixation or permeabilization of cells; therefore, compared with immunocytochemistry techniques using fixed cells, the likelihood of artifacts is reduced.
- Its tight, barrel-like structure protects the overall conformation of GFP protein and ensures that attachment of even large protein moieties does not affect its fluorescence capacity.

DISADVANTAGES OF GFP[16][17]

- The major disadvantage in using GFP as an in vivo marker protein is the lack of requirement of exogenous substrates or cofactors to produce the active fluorescent molecule.
- However GFP requires molecular oxygen to catalyze the post translational cyclization to form the proteins fluorophore. This requirement may be a problem when GFP is used as a reporter in biological systems where oxygen is limiting.
- GFP had one feature, that make it unsuitable for expression in organisms other than the jellyfish: The chromophore was formed by the cyclization of the peptide backbone between Ser-65 and Tyr-66. One or more converting enzymes were needed to change what was referred to as apoGFP to the fluorescent product **GFP AS AN ANALYTICAL TOOL.**

Yeast in fermentation

In fermentation the critical process is the conversion of carbohydrate source into ethanol with the prime involvement of an enzyme. The successful conversion of fermentation material into ethanol is possible only if the enzyme is maintained in a good condition till the entire process is complete. *Saccaromyces cerevisae, Escherchia coli, Lactococcus lactis, Hansenula polymorpha* have been widely used as the fermenting enzyme for the GFP to express in them. This expression will aid in detecting the stress condition, 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 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 eukaryotic and prokaryotic cells.[16] GFP expressed by the PyGFP3 plasmid and transformed into *Saccaromyces cerevisae* was used to detect the contamination of fermentation fed by the spoiled yeast. [20] With the help of an optic probe the expression of fluorescent protein was measured in a bioreactor during its on-line process.[21]

Other Applications

A Fusion Tags To Visualize Dynamic Cellular Events and to Monitor Protein Localization

B Reporter Gene

Green fluorescent protein has been extensively used as a reporter gene, especially in spatial imaging of gene expression in living cells.

C Photo bleaching to Investigate Protein Dynamics

Photo bleaching can be used to investigate protein dynamics in living cells. There are two methods based on photo bleaching: fluorescence recovery after photo bleaching (FRAP) and fluorescence loss in photo bleaching (FLIP). By mination illuminating an area with high intensity illumination (bleaching) and monitoring the recovery of the resultant fluorescence loss (FRAP), the relative mobility of the GFP chimera can be determined. FLIP can be used to study transport of GFP fusion proteins between different organelles by repeatedly bleaching an area and monitoring the loss of fluorescence from outside the area.[30]

D Metal Binding GFP Mutant As Metal Ion Biosensor

Wild-type GFP has a strong affinity for Cu(II), less for Ni(II), and negligible interactions with Zn(II) and Co(II). It contains 10 histidine residues, 5 of which are involved in secondary structures and are unlikely to bind metal ions. His 77, His 81, and His 231 are all within 7.5 Å of each other in the wild-type crystal structure of GFP (1EMF) and have been proposed as a possible site for metal interaction. Since metal ions in the vicinity of a chromophore are known to quench fluorescence in a distance dependent fashion, a metal-binding GFP mutant was designed as a potential in vivo metal ion sensor. [40]

E GFP To Monitor pH In vivo

Wild-type GFP and many of its mutants display pH-dependent fluorescent behavior and have been used to monitor pH in vivo. pH-sensitive mutants with p*K*as ranging from 6.15 to 7.1 have been reported. While traditional synthetic pH indicators have not been very effective at monitoring mitochondrial matrix pH, GFP-based pH indicators have successfully measured cytosolic, mitochondrial, and Golgi pH. EYFP has a p*K*a of 7.1 and has been used as a Golgi and cytosolic pH indicator. For organelles that are more acidic than the Golgi and cytosol, EGFP is used because EYFP is non fluorescent at thesepH. ECFP is less pH sensitive than either EYFP or EGFP and is rarely used.[42]

G As a marker

GFP has been used as a marker for tumor cells to illuminate tumor progression and allow for detection of metastases down to the single-cell level and as a whole-body optical imaging system in live mice. Caution should be used when applying GFP in low oxygen conditions (hypoxia) such as those found in tumor cells. Histone-GFP fusions have been designed that were sufficiently sensitive to visualize double minute chromosomes in vivo. Double minute chromosomes are paired chromatin bodies found in as many as 50% of human tumors but not found in normal chromosomes. A rapid cell based, functional assay for the screening of chemo preventive agents using GFP as a reporter gene has been developed.[43]

CONCLUSION

PAT have shown its importance in many of the process concerned with the pharmaceutical industry with no exception to the production of biological products, fermentation being of core importance in the production dextran antibiotics like penicillin and many others. One important tool to obtain a deeper process understanding is the use of modern real- time process sensors and analyzers. Usually industrially accepted optical probe for turbidity measurement has been adapted for on- line and real time fluorescence measurement in bioreactors. By tagging product proteins with fluorescent receptor molecules (eg.GFP) it was possible to quantify product concentration during a fermentation process with the new probe. GFP alone can be used in monitoring the activity of bacteria, yeast or enzyme in fermentation process. Monitoring of the raw material and the 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 in time but also reduces the risk of recalling of product from the market.

REFERENCE

1. SunitaOgale et al., Int. J. Res. Pharm. Sci., 2013; 4(3): 350-353.
2. Int. J. Pharm. Sci. Rev. Res, 2012; 17(1): 13, 57-65.
3. Tsien, R.(1998) Annu. Rev. Biochem., 1998; 67: 509-544.
4. Morise H, Shimomura O, Johnson FH, Winant J. "Intermolecular energy transfer in the bioluminescent system of Aequorea",PUBMED, Biochemistry, 1974; 13(12): 2656-2662.
5. Ibtesam A, Saeeds, Salman Ashraf, "Denaturation studies reveal significant differences between GFP and blue fluorescent protein", International Journal of Biological Macromolecules, October 2009; 45(3): 236-241.
6. Ormö, M. et al. *Science.*, 1966; 273: 1392-1395.
7. Brejc, K. et al. *Proc. Natl. Acad. Sci. USA.*, 1997; 94: 2306.
8. Ghosh I, Hamilton AD, Regan L. Antiparallel leucine zipper-directed protein reassembly: applicationto the green fluorescent protein. J Am Chem Soc., 2000; 122: 5658–5659.
9. Kent KP, ChildsW, Boxer SG. Deconstructing greenfluorescent protein. J Am Chem Soc., 2008; 130: 9664–9665.
10. Schwede, T. F.; Retey, J.; Schulz, G. E. *Biochemistry.*, 1999; *3*5355-5361.
11. Donnelly, M.; Fedeles, F.; Wirstam, M.; Siegbahn, P. E. M.;Zimmer, M. *J. Am. Chem. Soc.*, 2001; *123*: 4679-4686.
12. Prasher, D. et al. *Gene.*, 1992; 111: 229-233.
13. Shimomura, O. FEBS Letters., 1979; 104: 220-222.

14. Heim, R. et al., *Proc. Natl. Acad. Sci. USA.,* 1994; 91: 12501-12504.
15. Cubitt, A.B. et al. *Trends Biochem. Sci.,* 1995; 20: 448-455.
16. Chalfie M TuY, Euskirchen G, Ward W W, Prasher DC."Green Fluorescent Protein as a marker for gene expression" Department of biological Sciences, Columbia University, NewYork, NY10027., Science., 1994 Feb11; 263(5148): 802-5.
17. Wang,S.X. and Hazelrigg, T. Nature., 1994; 369: 400-403.
18. Kummer AD, Kompa C, Lossau H, Pollinger-DammerF, Michel-Beyerle ME, Silva CM, Bylina EJ, ColemanWJ, Yang MM, Youvan DC. Dramatic reduction in fluorescence quantum yield in mutants of green fluorescent protein due to fast internal conversion. Chem Phys., 1998; 237: 183–193.
19. Megley CM, Dickson LA, Maddalo SL, Chandler GJ, Zimmer M. Photophysics and dihedral freedom of the chromophore in yellow, blue, and green fluorescent protein. J Phys Chem B., 2009; 113: 302–308.
20. Luiz H G, Keila MRD, Felipe GA, Ana MBG, Flavio CAT. "A vector carrying The Gfp Gene (Green Flu-orescent Protein) as a Yeast Marker for FermentationProcesses", Sci. agric., Oct. / Dec. 2000; 57(4): 713-16.
21. Randers L E, Albano CR,SipiorJ,Bentley WE, RaoG.On line green fluorescent protein sensor with LED excitation,Biotechnology and Bioengineering, September, 1997; 55(6): 921-26.
22. Tobias B , Odermatt RP, Ledergerber P "Exploiting Fluorescent Reporter Molecule for Process Analytical Technology (PAT)", 2009,171-173.In CHIMIA International Journal for Chemistry, B Sonnleitner, Sensorik in der Bioprogresstechnik, 2003; 63(3): 1-3.
23. Lakowicz JR. "Principles of Fluorescence Spectroscopy", Newyork. Plenum Press; 2nd edition, 1983; 233.
24. Bartolome A J, Ulber R, Scheper T, Saji E, Belkins."Ge-notoxicitymonitoring using a 2D – spectroscopic GFP wholecellbiosensing system", Sensors and Actuators B: Chemical, Elsevier, March 2003; 89(1): 27-32(6).
25. Tavare JM, Fletcher LM, and Welsh GI "Using Green Fluorescent Potein to study intracellular signaling" Journal of Endocrinology, 2001; 170: 297-306.
26. Prescott, M.; Nowakowski, S.; Nagley, P.; Devendish, R. *Anal.Biochem.,* 1999; *273:* 305-307.
27. Li, X.; Zhao, X.; Fang, Y.; Jiang, X.; Duong, T.; Fan, C.; Huang,C.-C.; Kain, S. *J. Biol. Chem.,* 1998; *273:* 34970-34975.
28. Bardeen, C. J.; Yakovlev, V. V.; Squier, J. A.; Wilson, K. R. *J.Am. Chem. Soc.,* 1998; 13023-13027.
29. Miyawaki, A.; Llopis, J.; Heim, R.; McCaffrey, J. M.; Adams, J.A.; Ikura, M.; Tsien, R. Y. *Nature.,* 1997; *388:* 882-887.
30. Griesbeck, O.; Baird, G. S.; Campbell, R. E.; Zacharias, D. A.; Tsien, R. Y. *J. Biol. Chem.,* 2001; *276*: 29188-29194.
31. Jensen, K. K.; Martini, L.; Schwartz, T. W. *Biochemistry.,* 2001; *40:* 938-945.
32. Nock, S.; Spudich, J. A.; Wagner, P. *FEBS Lett.,* 1997; *414:* 233-238.
33. Schwille, P.; Kummer, S.; Heikal, A. A.; Moerner, W. E.; Webb,W. W. *Proc. Nat. Acad. Sci. U.S.A.,* 2000; *97*: 151-156.
34. Zhu, M.; Fahl, W. E. *Anal. Biochem.,* 2000; *287*:210-217.
35. Ando R. et al. *Proc. Natl. Acad. Sci. USA.,* 2002; 99: 12651-12656.
36. Baird, G.S. et al. *Proc. Natl. Acad. Sci.,* 2000; 97: 11984-11989.
37. Cormier, M.J. et al. *J. Cell Physiol.* 1973; 81: 291-297.
38. Davenport, D. and Nicol, JAC. *Proc. R. Soc. London,* SerB., 1955; 144: 399-411.
39. Elf, J. et al. *Science.,* 2007; 316: 1191-1194.
40. Ellenberg, J. et al. *Biotechniques.,* 1998; 25: 838-842.
41. Fradkov, A.F. *FEBS Letters.,* 2000; 479: 127-130.
42. Ghaemmaghami et al. *Nature.,* 2003; 16: 737-741.
43. Gross, L.A. et al. *Proc. Natl. Acad. Sci. USA.,* 2000; 97: 11990-11995.