International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 6 Nov-Dec 2022, pp: 1687-1697www.ijprajournal.com ISSN: 2456-4494

A review on Rapid Microbiological Methods

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Submitted: 15-12-2022 Accepted: 26-12-2022

ABSTRACT:
A Rapid Microbiological Method is an antidote to traditional compendial microbiological methods with its own assets and liabilities. As the intricacy of the drugs elevated in the pharmaceutical and biotechnology domains, these drugs requires critical, complicated handling and monitoring while ensuring the microbial quality. For example, microbial quality of Positron Emission Tomography drugs is defined by sterility test and Bacterial endotoxin test[1]. Sterility Testing can be performed by Adenosine Tri Phosphate Bioluminescence and Membrane Laser Scanning Fluorescence Cytometry as an alternative to the compendial Membrane Filtration method as they report results quickly by means of reducing the incubation period from 14 days to 5 days. On the other hand, Bacterial Endotoxin Test can be performed by Monocyte activation test and Recombinant factor C assay in preference to the classical Limulus Amebocyte Lysate Gel Clot Method as these techniques are highly sensitive, accurate and reproducible. The main restraint for afore mentionedmethods in performing sterility test is that they are non-CFU based Rapid Microbiological Methods whereas membrane filtration is a CFU based classical method thereby ensuing a problem in establishing the acceptance criteria and equivalence during validation process[2]. Hence, this review focusses on the comparison and contrast of different aspects related to traditional and rapid microbiological techniques which may extend an ultimate solution for the existing limitations towards Rapid Microbiological Methods so as to acquire regulatory compliance.

INTRODUCTION:

The complexity and diversity of diseases in human beings demanded the need to discover the drugs starting from over the counter drugs, antibiotics to the orphan drugs [3]. This created a rapid fire between several pharmaceutical and biotechnological companies worldwide towards the research of quality assurance and quick product release of the respective drugs into the market. This led to the advent of Rapid Microbiological methods which availed its own importance by virtue of its notable attributes such as sensitivity and accuracy[4]. RMM's are highly validated and are not time and labor intensive[5]. Over the centuries in the field of microbiology, traditional methods adopted by Louis Pasteur and Robert Koch had been followed till date and were being updated at lumbering pace[6].

Basically any sample quality, by employing microbiological methods can be defined applying three types of tests namely qualitative, quantitative and identification tests[7]. Comprehensively, these tests are detection of presence or absence of microorganisms in the sample, Enumeration of microorganisms in the sample, Identification of specified pathogens in the sample[8]. Different RMM techniques have been developed to perform earlier termed microbiological methods and have to be selected depending upon vital quality feature of the product, validation prerequisites and GMP guidelines[9]. Practically, RMM's are classified as growth-based, viability-based, cellular component-based, Optical Spectroscopy, nucleic acid-based and Micro-Electro-Mechanical Systems (MEMS) based technologies[7] which is as shown in Figure 1.

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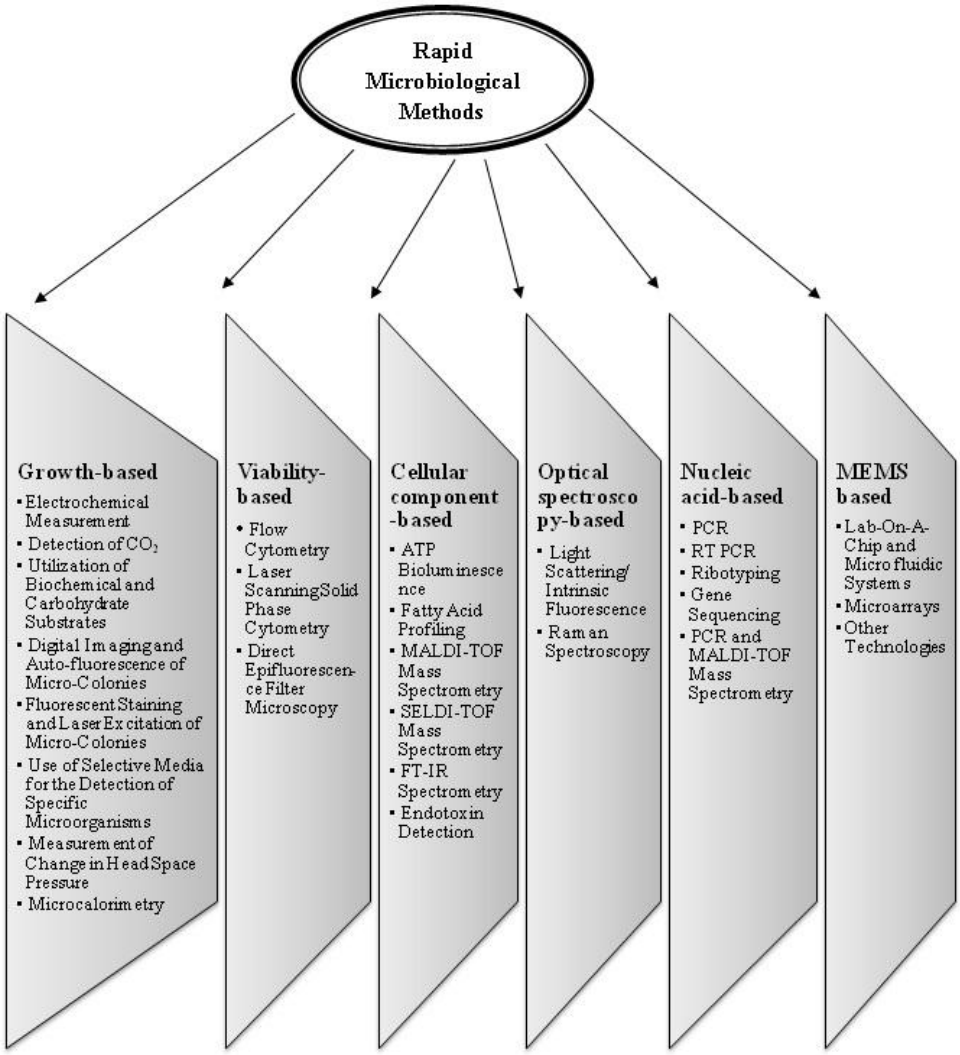

Figure 1 Empiricalclassification of Rapid Microbiological Methods[7]

Traditional compendial methods have not been upgraded because of several reasons such as lack of awareness and vacillation on latest RMM's, validation difficulties in setting up the acceptance criteria, lack of equivalence between traditional and RMM methods and the affordability by many companies economically[10]. Hence, this review comprises of extensive elucidation on each and every RMM technique in a comparative manner and an elaborate illustration on statistical and economical significance of RMM's have been incorporated to create and intensify the awareness, positive dominance and necessity of RMM in the field of advanced analytical Microbiology so as to increase the confidence level to adapt the RMM and also may lead to several solutions for the hurdles exists in the present day research of RMM's.

CELLULAR COMPONENT-BASED RAPID MICROBIOLOGICAL METHODS:

ATP Bioluminescence:

Bioluminescence is a natural trait exhibited by distinct organisms irrespective of their cellular complexity, for their defense and survival[11]. This phenomenon in firefly is due to the crucial participation of ATP molecule in the reaction of converting the energy into light implying the term 'ATP Bioluminescence' which is explicitly displayed in Figure 2. Consequently, the intensity of light is directly proportional to the ATP utilized in the reaction[12].Further, ATP detection sensitivity has been improved rapidly using Adenylate Kinase[13]. These two facts form the basis for rapid ATP detection devices in the field of microbiology to determine the microbial

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contamination and concentration. In this context, ATP bioluminescence has been applied prominently in sterility testing of pharmaceutical products rather than conventional methods[14]. The implementation of above technique is highlighted upon the recovery of even slow growingPropionibacteriumacneswithin 4-5 days instead of usual 14 days[15]. Furthermore, Bacterial bioluminescence is another exemplarwith several applications in which $FMNH_2$ and NADH are the prime contributors[16, 17]which is displayed precisely in Figure 3.

ATP bioluminescence Vs Bacterial bioluminescence:

S. No:	ATP Bioluminescence	Bacterial Bioluminescence
1	ATP bioluminescence is eukaryotic trait[18].	Bacterial Bioluminescence is prokaryotic triat[18].
2	The well understood eukaryotic bioluminescent systems are firefly systems particularly Photinus pyralis, sea pancyRenillareniformis, marine copepodGaussia princeps[19].	Prokaryotic systems such asPhotobacteriumluminescens, Vibrio fischeri, V. harveyi, P. phosphoreumandP. leiognathiare the efficient comprehend systems [19].
3	The cofactors involved in the firefly bioluminescence reaction are ATP and Mg^{2+} in the presence of molecular oxygen[16].	$FMNH^2$ and NADH are the cofactors that contribute in the bacterial bioluminescence in addition to the molecular oxygen[16].
4	Firefly luciferase is a Photinus-luciferin 4-monooxygenase of enzyme class EC 1.13.12.7[20].	Bacterial luciferase belongs to the Flavin dependent monooxygenase family with enzyme class EC 1.14.13.8[21].
5	FLuc is the firefly luciferase[22]	The operon that gives off enzyme, substrate and other necessary compounds for the respective bioluminescent reaction is lux operon[23].
6	Luciferase mediated light reaction releases the photon energyin the form of yellow-green light at a wavelength of 550-570 nm[19].	Blue-green light at a wavelength of 490 nm is emitted as the resultant of the luciferase catalyzed reaction[19].
7	Consistent emission of light even at lower cell concentrations made this ATP bioluminescence a powerful tool for different applications[24,25].	The light emission will be declined after reaching the stationary growth phase of organisms[26].
8	Firefly luciferase has better activity at temperatures above 22.5°C.Hence,it is suitable for the detection of organisms that require optimum growth temperatures[27].	As Vibrio luciferases are stable even at lowtemperatures thereby extending theapplication to the lower growth temperature favored organisms. On the other hand, P.luminescens luciferases have notable stability at higher growth temperatures[19].
9	This bioluminescence can be preferably applied to perform in vitro experiments. On the contrary, it can be extended to in vivo experiments owing to the fact that firefly can luminescent red light	This phenomenon is better competent towards invivo experiments because it does not require external addition of

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	under acidic pH conditions, which has higher tissue penetration capacity[24, 25].	substrate unlike firefly bioluminescence[24, 25].
10	Transformation of luc genes is difficult due to its large size which became a remarkable limitation to the successful application of this technique towards in vivo studies[28].	Transformation of lux genes is quite easy and successful comparitively[29].

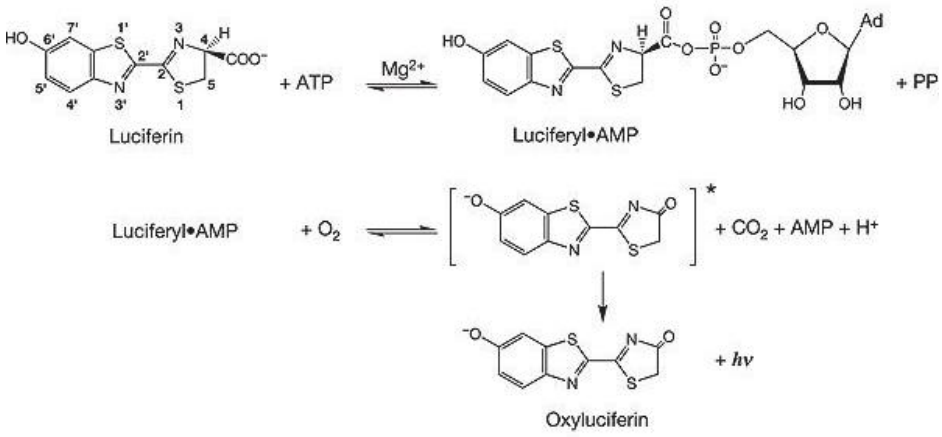

Figure 2 Firefly Luciferase mediated ATP Bioluminescence reaction[30]

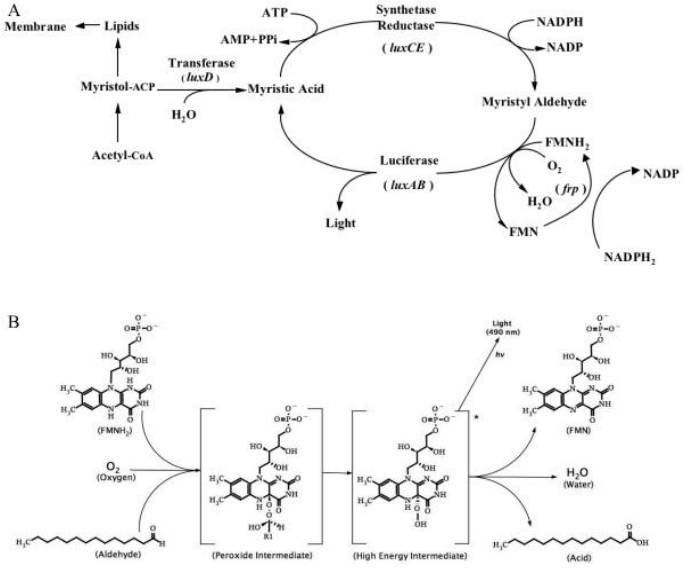

Figure 3Bacterial Luciferase mediated Bioluminescence reaction[25]

Irrespective of highlights and challenges of ATP bioluminescence, it has been raised as current rapid testing tool ofand for science and technology[14].The spotlight of current research of this technique has been pivoted on the identification of divergent bioluminescent mechanisms[31], development of different luciferin analogs and luciferase mutants[32] and validation perspectives[33]. Approximately, 30 diverse bioluminescent mechanisms have been reported till date[31]. In recent years, bioluminescence similar tothat of firefly in terms of ATP dependence have been identified in earthworm species namely Fridericia heliotaalong with the recognition of bioluminescence in higher fungi which needs only oxygen excluding any other cofactors with the involvement of a luciferin precursor, 'Hispidin'[31]. Synthetic luciferin substrate molecules such as aminoluciferins[34], dehydroluciferin, decarboxy- and dehydroxyluciferins, 5,5-dimethylluciferin, synthetic luciferin compounds with replaced benzothiazole fragments, hydrazide D-

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luciferin[35]and brominated luciferins[36]have been innovated.These synthetic luciferin analogues have depicted eclectic peculiarities such as emission of intense light with increased wave length, pH stability, thermostability and improved specific activity when compared to D-luciferin,dependingupon the mutant and engineered luciferases[32]. For instance, brominated luciferins exhibited intense red lightemissionthanD-luciferin with high tissue penetrating capacity by means of recombinant firefly luciferase[36].

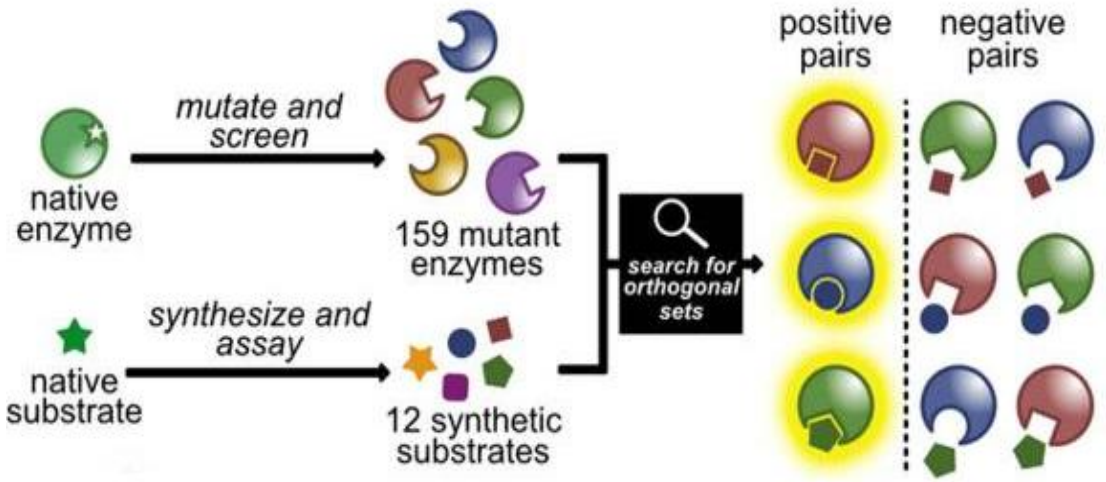

Figure 4Schematic for obtaining orthogonal luciferin analogues-mutated enzyme pairs[37]

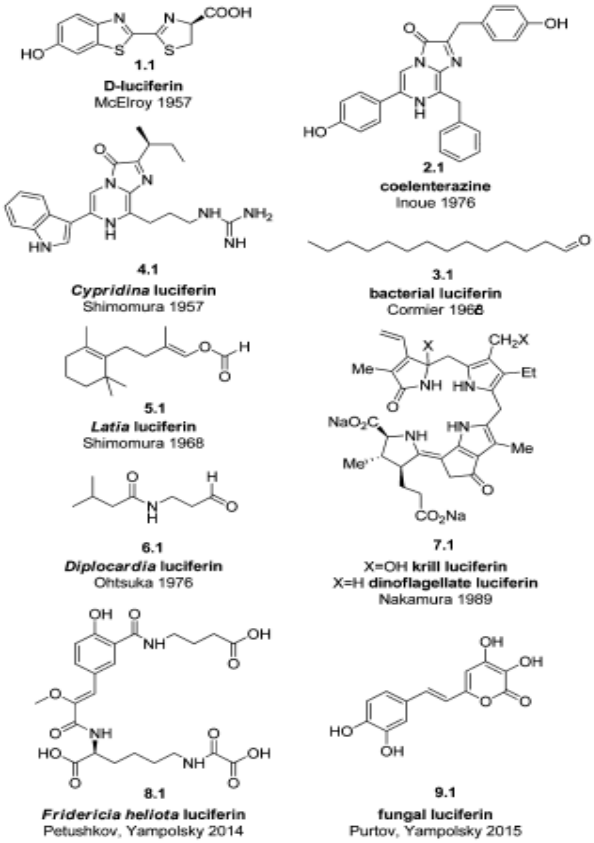

Figure 5Meticulously understood 9 luciferin molecules till date[32]

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Validation perspectives:

The majorconstraintduring validation of ATP bioluminescence is the interfering components affecting ATP-light reaction[38]. Robustness of ATP detection systems has been affected by the stressed environment under which the validation has to be performed. Rapid Sterility test medium which is anagar instead of broth[39]and sample-bearing swabs have been employed to overcome the above problem[33]. Concurrently, the above agar medium has positive impact on the specificity of the rapid method by recovering a wide variety of stressed microorganismsthan the usual liquid media[39]including non-cultivable eukaryotic integrants[38]. There has beennonoticeablestatistical significanceforLimit of detection between conventional and rapid sterility tests[39]. Limit of detection is very low for pure cells. Synchronously, gram positive cells have showed an appreciable limit of detection than the gram negative bacterial cells. The sensitivity towards gram negative cells can be enhanced partially by sonication[33].Low level ATP concentration is the concern in deviating accuracy and precision of RLU valuesduringsterility tests[33, 40].

Fatty acid profiling:

Fatty acid profiling has been emerged as a rapid method for the spotting of numerous microorganisms in which fatty acids serves as means of identification[41], as lipids are prominent cellular components[42]. The initial step in profiling the fatty acids is the extraction of lipids from cells followed by deriving of fatty acids from lipids and analyzing the samples employing appropriate analytical techniques by means of modifying them to form fatty acid methyl esters (FAME's)[43]which is clearly depicted in the figure 6.The fatty acids have been converted to FAME's to make them volatilethus enabling them to undergo gas chromatographic analysis conveniently, along with the flexible free fatty acid detection.(Macedo LFA, Lacerda ECQ, Silva RR, Simionato JI, Pedrao MR, Coro FAG et al. Implications of Method Chosen for Analysis of Fatty Acids in Meat: A Review. Am J Agric Biol Sci. 2012;7(3):278-84.)

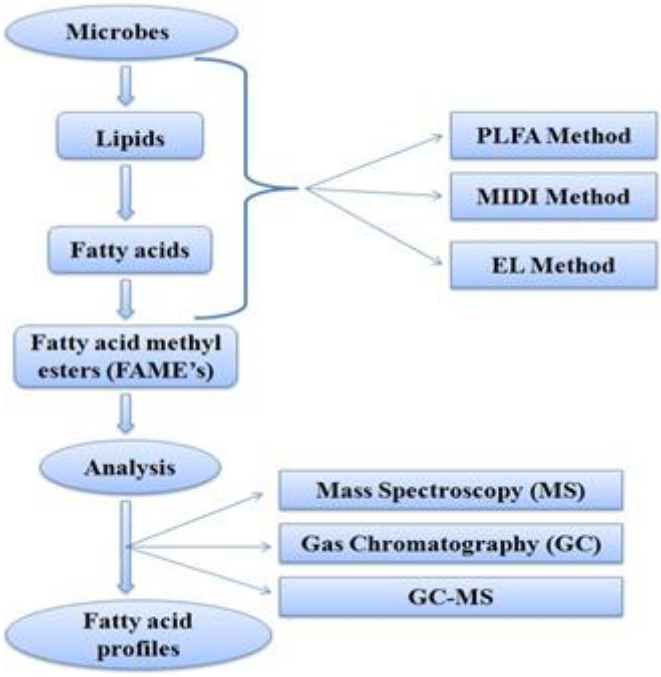

Figure 6 General schematic of fatty acid profiling

Three methods have been employed to derive fatty acid methyl esters from the microbial samples as mentioned in figure 6. The brief description of the above three methods are as follows:

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PLFA Method:

Phospholipids gained interest as they can act as a measure of living cells. Thus the phospholipids have been targeted for estimating the viable biomass. Also, taxonomical classification and estimating physiological stress of microbes have been possible using Phospholipid Fatty Acid (PFLA) method[44]. PLFA analysis comprises of four basic steps namely: 1) Lipid extraction in which single phase solvent extraction has been employed.2) Fractionation of total lipid by means of silicic acid chromatography, 3) Deriving Fatty Acid Methyl Esters (FAME's) upon methanolic transesterification and 4) Separation and quantification of FAME's[45]. The critical limitation of PLFA analysis is towards the mixed cultures. This method cannot identify specific microbes till its species level in mixed cultures and thereby can be applicable only to estimate the overall biomass communities present in the sample[46].The traditional extraction method has become efficient by adoptingtwo phase extraction which is also known as Bligh and Dyer method involved with mild treatment with chloroform, methanol and water[47]. Further, lipid extraction has been still improved by means of citrate buffer instead of phosphate buffer, acetate buffer, tris and water[48]. On the other hand, a new fractionation column has been developed competitive to the silicic acid column which is well known as solid phase extraction column[49]. Later, PLFA method has been out-turned to be efficient in terms of time, capital and reproducibility upon scaling down the volumes of both the sample and other auxiliary compounds involved[50]. PLFA analysis has been a notable technique in identifying both fungal and bacterial communities using the same sample[51]. PLFA technique has become more reliable even to distinguish substrate level utilization differences in the microbial communities particularly in the soil samples by labelling the substrates using stable isotopes such as ^{13}C[46, 52].

MIDI Method:

Basically,MIDI method involves five basic steps, that are1) Harvesting of microbial cells, 2) Saponification by applying sodium hydroxide, methanol and distilled waterin appropriate proportions 3) Methylation by means of hydrochloric acid and methyl alcohol, 4) Extractionusing hexane and methyl tert-butyl ether and 5) Washing with sodium hydroxide[53]. MIDI method when compared to PLFA method has this unique step of saponification,is for better extraction of fatty acids to avoid any interfering compounds during further analysis[54]. MIDI method can identify microorganisms till the species level in an effective manner than the PLFA method even within small amounts of sample and can perform whole lipid analysis.Also, MIDI method can identify hydroxy fatty acidswhich are the signature fatty acids for certain microbes such as acetic acid bacteria, Pseudomonas sp, Enterobacteriaceae and Dimethylacetalswhich have been the evidence for stringent anaerobic conditions respectively unlike PLFA method[55].

S. No	Types of Fatty Acids (FAs)		Signature FAs for
1	Phospholipid FAs		Microbial biomass
2	Ester-linked polyunsaturated FAs		Eukaryotes; cyanobacteria
3	Non-ester linked unsubstituted FAs		Clostridium; eukaryotes
4	Ester-linked saturated FAs	Straight-chain FAs	Eukaryotes; widespread
		Saturated straight chain longer than 20C atoms. For example 20 : 0, 21 : 0, 26 :0 etc.	Eukaryotes, mosses, higher plants
		FAs containing cyclopropyl ring	Gram negative: Rhodospirillum, Cromatium/Legionella; Gram positive: Clostridium, Bifidobacterium
		Position of methyl branching is iso or anteiso	Gram positive, Gram negative: Cytophaga, Acetobacter, Flavobacterium
		Branched-chain FAs (position of methyl branching is unknown)	Gram positive: actinomycetes

International Journal of Pharmaceutical Research and Applications
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		Methyl branching on 10th C atom	Actinomycetes, sulphate reducer
5	Ester-linked monounsaturated FAs	Vaccenic type (anaerobic desaturase pathway)	Gram-negative aerobes, strictly anaerobes
		Oleic type	Gram positive, widespread; Methane-oxidising bacteria, Type I methanotrophs
6	Ester-linked hydroxy FAs	Hydroxy substitution at position 2 nearest to carboxyl end	Pseudomonas, Gram negative, actinomycetales
		Hydroxy substitution at position 3 nearest to carboxyl end	Mycobacterium
		Hydroxy substitution at position 3 nearest to aliphatic end	Fungi
7	Non-ester-linked hydroxy substituted FAs	Hydroxy substition at position 2 nearest to carboxyl end	Sphingomonasspp., Candida spp.
		Hydroxy substitution at position 3 nearest to carboxyl end	Bacteroides/Flavobacterium
		Beta-OH alpha-branched	Mycobacterium, Nocardia
8	Hydroxy substituted fatty acids localised in outer Membrane (lipopolysaccharides)	Hydroxy substitution at position 2 nearest to carboxyl end	Gram negative, fungi
		Hydroxy substitution at position 3 nearest to carboxyl end	Gram negative except Eikanella, Arthrobacter, fungi
		Dicarboxylic fatty acids	Plants

Table 2: Signature fatty acids for different living organisms[56]

Conclusion: A Rapid Microbiological Method is an antidote to traditional compendial microbiological methods with its own assets and liabilities. As the intricacy of the drugs elevated in the pharmaceutical and biotechnology domains, these drugs requires critical, complicated handling and monitoring while ensuring the microbial quality. For example, microbial quality of Positron. Perhaps more than in any other sector of industrial microbiology, the routine microbiological testing carried out by the pharmaceutical manufacturing industry is determined by regulatory requirements. In this review we have discussed various microbiological methods, along with their advantages and disadvantages.

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