CHAPTER 2

THEORIES

2.1 Theoretical Background

2.1.1 Microbial community dynamic

Currently, conventional cultivation-dependent microbiological techniques fail to give any indication of biodiversity in biodegradable plastics. Therefore, molecular techniques based on 16S rRNA gene were successfully applied to microbial ecology research. Consequently, biodiversity was evaluated with three rRNA-based complementary methods: denaturing gradient gel electrophoresis (DGGE), Ribosomal intergenic spacer analysis (RISA) and construction of 16S rRNA gene libraries (Figure 2.1).

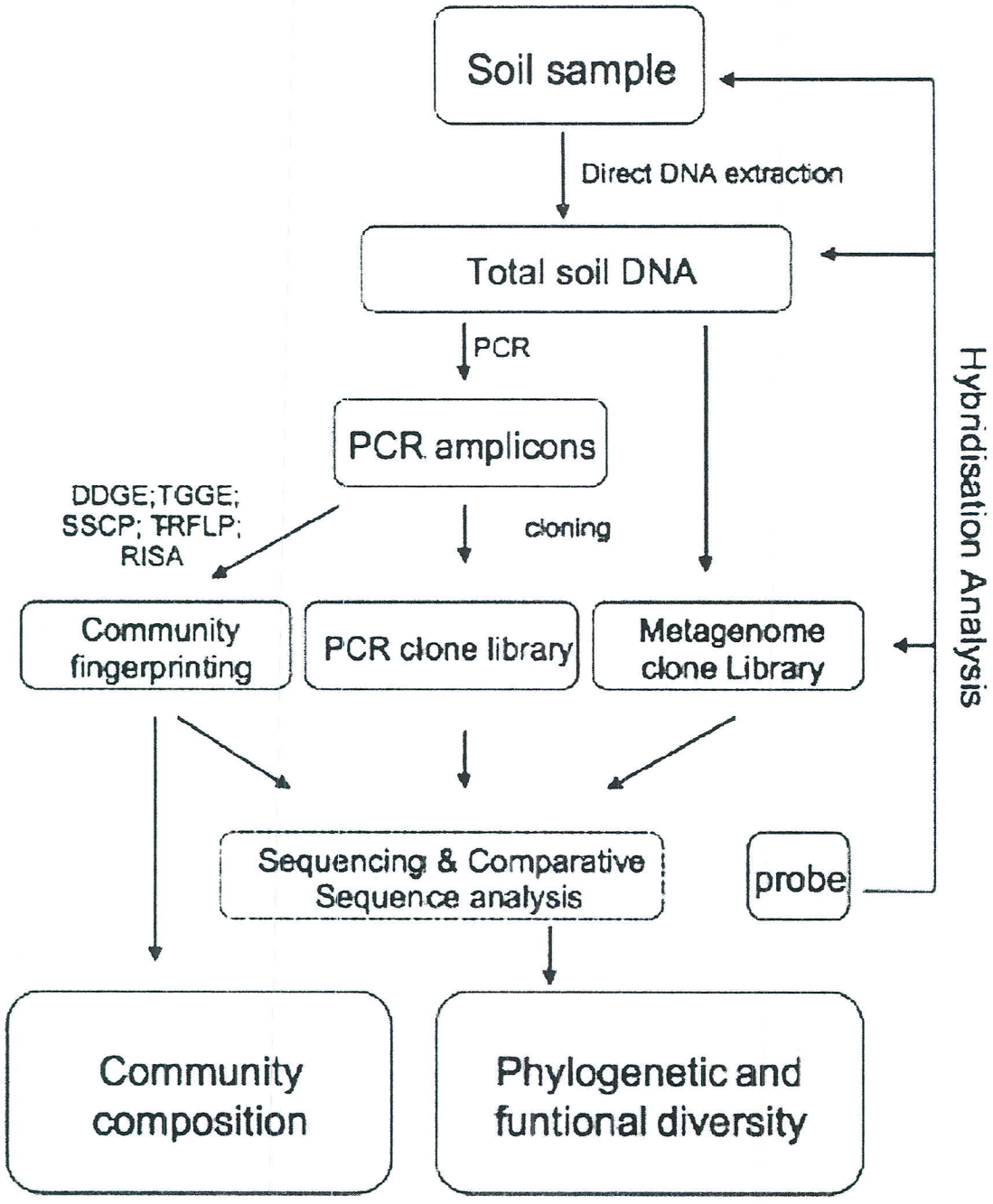

Figure 2.1 Flowchart of the different steps in the study of the structure and function of microbial communities. Culture independent molecular methods for the analysis of microbial communities comprised of PCR: polymerase chain reaction; DGGE: denaturant gradient gel electrophoresis; TGGE: temperature gradient gel electrophoresis; SSCP single strand conformation polymorphism; T-RFLP: terminal restriction fragment length polymorphism; RISA: ribosomal intergenic spacer analysis (García, 2008).

2.1.2 Polymerase chain reaction (PCR)

PCR is a molecular genetic technique for making multiple copies of a gene using a sample of DNA. The technology was good enough to make multiple copies from one single copy of the gene found in the sample. A method for amplifying segments of DNA, by generating multiple copies using DNA polymerase enzymes under controlled conditions. As little as a single copy of the DNA segment or gene could be cloned into millions of copies, allowing detection using dyes and other visualization techniques. The process of PCR had made it possible to perform DNA sequencing and identify the order of nucleotides in individual genes. The basic PCR procedure includes 3 steps repeated 20 - 30 times (Figure 2.2).

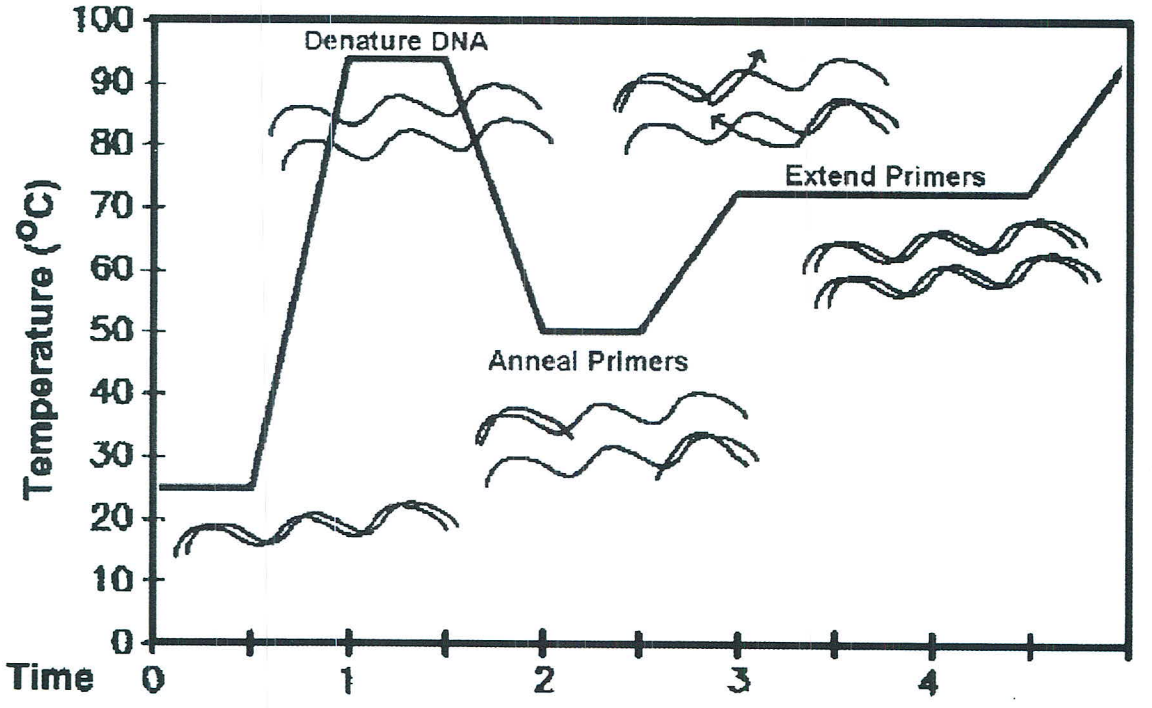

Figure 2.2 Polymerase Chain Reactions (PCR).The process consisted of: 1) Denaturing: heating the double stranded DNA (to 90-95°C) to separate the strands, 2) Annealing: lowering the temperature to allow primers to bind single stranded DNA, 3) Extension: the DNA polymerase adds nucleotides to the growing strands

2.1.3 Denaturing gradient gel electrophoresis (DGGE)

In order to understand the effects that different wastewater may have on the microbial consortium present in different UASB granule, the polymerase chain reaction

(PCR) based on DGGE technique and sequence analysis were used to fingerprint and identify the dominant bacteria in UASB granules. The DGGE had been shown to detect differences in the melting behavior of small DNA fragments (200-700 bp) that differ by as little as a single base substitution. When a DNA fragment was subjected to an increasingly denaturing physical environment, it partially melts. As the denaturing conditions became more extreme, the partially melted fragment completely dissociates into single strands. Rather than partially melting in a continuous zipper-like manner, most fragments melt in a step-wise process. Discrete portions or domains of the fragment suddenly become single-stranded within a very narrow range of denaturing conditions. The rate of mobility of DNA fragments in polyacrylamide gels changed as a consequence of the physical shape of the fragment. Partially melted fragments migrated much more slowly during electrophoresis through the polyacrylamide matrix than completely double-stranded fragments.

When a double-stranded fragment electrophoreses into a gradient of increasingly denaturing conditions, it partially melts and undergoes a sharp reduction in mobility because it changes shape. In practice, the denaturants used were heat (a constant temperature of 60°C) and a fixed ratio of formamide (ranging from 0-40%) and urea (ranging from 0-7 M). The position in the gradient where a domain of a DNA fragment melts and thus nearly stops migrating was dependent on the nucleotide sequence in the melted region. Sequence differences in otherwise identical fragments often caused them to partially melt at different positions in the gradient and therefore 'stop' at different positions in the gel. By comparing the melting behavior of the polymorphic DNA fragments side by side on denaturing gradient gels, it was possible to detect fragments that had mutations in the first melting domain.

The usefulness of DGGE in the analysis of microbial communities rested on the assumption that different sequences would migrate to different positions in DGGE gels (Muyzer *et al.*, 1998). The DGGE separates DNA sequences based on their melting behavior (Lerman *et al.*, 1987) and simulations showed that 95% of single base sequence differences would be detected by this method (Myers *et al.*, 1985). The ability of the technique to separate genomic sequences differing by more than one base has rarely been examined, although it has been suggested the that detection of multiple sequence differences may be more difficult (Lyons, 1994).

Although there were many advantages to using PCR-DGGE for microbial community analysis, method limitations must be recognized for correct result

interpretation. The greatest challenge in performing DGGE and attaining reproducible results was minimizing variation between gel gradients (Fromin *et al.*, 2002). It was very difficult to exactly reproduce these gel gradients, which must be kept in mind when performing between-gel comparisons. The inclusion of DGGE markers on all gels had been used to aid between-gel comparisons and to assess gradient variations. Ideally, a marker should be chosen with a sufficient number of bands to span the entire gradient, since there was variation within a gradient.

Another limitation of DGGE was that a complex community (e.g. soil and/or mixed cultures) may be comprised of numerous populations (from >100 and possibly >108) in relatively equivalent proportions, thus resulting in a smear of bands, which made it difficult to identify individual populations (Nakatsu *et al.*, 2000). It was still possible to qualitatively state that two communities were different if the smear of bands looks different; however, the converse may not be true. The number of bands observed in a profile cannot be interpreted to be the exact numbers of populations in a community. In some cases, investigators had found that a single laboratory isolate could produce multiple bands by DGGE (Nübel *et al.*, 1996; Satokari *et al.*, 2001), and conversely, a single band may represent multiple populations (Yang *et al.*, 2000). Combining DGGE profiling with other techniques, such as sequencing of bands or hybridization with probes can reduce the ambiguity of band identification (Stephen *et al.*, 1998). Nevertheless, the method could provide an estimate of richness that enables researchers to determine subsequent analyses that could be conducted and a means of choosing samples representing unique or representative communities (Joynt *et al.*, 2006).

2.1.4 Ribosomal Intergenic Spacer Analysis (RISA)

The RISA was also a DNA-based method for microbial community analysis. It involved PCR amplification of a region of the rRNA gene operon between the small (16S) and large (23S) subunits termed the intergenic spacer region. By using oligonucleotide primers targeted to conserved regions in the 16S and 23S genes, RISA fragments could be generated from the dominant bacteria in an environmental sample. While the majority of the rRNA operon served a structural function, the taxonomic value of the intergenic spacer region lies in the significant heterogeneity in both length and nucleotide sequence. In RISA, we exploited the length of heterogeneity of the intergenic spacer region between 150 bp and 1,500 bp with the majority of the intergenic spacer region lengths being between 150 bp and 500 bp (Fisher *et al.*, 1999).

The RISA protocol for bacterial community analysis described here was based on Borneman and Triplett (1997) and Yin *et al.* (2000). It had also been used for fungal community analysis (Ranjard *et al.*, 2001). The RISA technique was not famous, although many researchers used in order to compare bacterial community structure and diversity. For example, Benizri *et al.* (2005) compared the bacterial community structure in healthy and sick soils, and evaluated the possible role of cyanide, culture-independent method as RISA was used. Additionally, the RISA method was used for studying changes in microbial community structure during adaptation towards PHA production (Ciesielski *et al.*, 2009).

RISA has following features compared to DGGE:

1. The amplified region is longer (positions 23R to 1406F, i.e. 1383 bp).
2. No GC tail is attached to the forward primer.
3. The species differ in the length of the amplified region.
4. The species are separated according to the length of the region in an agarose or an acrylamide gel. The acrylamide gel may give a better resolution than the agarose gel.

In some cases, samples that cannot be amplified for DGGE can be amplified for RISA. This may be due to the fact that the primers for RISA do not contain a GC tail, which is more difficult to amplify.